AD_____

Award Number: W81XWH-11-2-0110

TITLE: Novel Compounds From Shark and Stingray Epidermal Mucus With Antimicrobial Activity Against Wound Infection Pathogens

PRINCIPAL INVESTIGATOR: Carl A. Luer, Ph.D.

CONTRACTING ORGANIZATION: Mote Marine Laboratory, Inc.

Sarasota, FL 34236-1096

REPORT DATE: March 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE: March 2014	**2. REPORT TYPE:** Annual Report	**3. DATES COVERED** 15 February 2013 – 14 February 2014

4. TITLE AND SUBTITLE: Novel Compounds From Shark and Stingray Epidermal Mucus With Antimicrobial Activity Against Wound Infection Pathogens

5a. CONTRACT NUMBER

5b. GRANT NUMBER: W81XWH-11-2-0110

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Carl Luer, Ph.D., PI; Catherine Walsh, Ph.D., Co-PI; Kimberly Ritchie, Laura Edsberg, Ph.D., Co-PI; Jennifer Wyffels, Ph.D., Co-PI; Vicki Luna, Ph.D., Co-PI; Ashby Bodine, Ph.D., Co-PI
E-Mail:caluer@mote.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mote Marine Laboratory
Sarasota, FL 34236-1096

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A protective secretion produced by epidermal mucus cells in stingrays is being investigated to understand its role in wound healing and to identify mucus-associated antimicrobial compounds with the potential for development into novel therapeutics to treat wound infection pathogens. Mucus from cownose rays (*Rhinoptera bonasus),* Atlantic stingrays (*Dasyatis sabina*), devil rays (*Mobula hypostoma*) and clearnose skates (*Raja* eglanteria) contains proteins in the aqueous supernatant and viscous pellet, as well as symbiotic bacteria. While fresh mucus extracted with a magnesium salt of trifluoroacetic acid possesses low but measurable antimicrobial activity, symbiotic bacteria isolated from the mucus consistently demonstrate antibiotic activity in both primary and secondary screens against pathogenic bacterial strains. Many of these strains are active against either MRSA or VRE. Experimental wounding studies result in wounds that heal without infection or inflammation. While a healing timeline based on gross physical appearance of wounds suggests gradual healing over several weeks, histology of biopsied wounds from day 28 and earlier reveals that day 2 wound beds are already covered with a thin epidermis containing mucus cells and a clearly identifiable basal layer over a developing but unstructured dermis.

15. SUBJECT TERMS
stingray epidermal mucus, antimicrobial activity, wound healing, wound infection pathogens, bacterial symbionts, culturable bacterial isolates

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	41	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION:

Combat wounds, particularly blast wounds, are highly susceptible to infection and delayed healing. As a result, U.S. military caregivers are constantly seeking new antibiotics to treat soldiers wounded in combat arenas as well as those recovering in hospitals. Animal models of rapid wound healing can provide significant insights into novel approaches of treating combat wounds. There are numerous examples of remarkable wound healing in sharks and their skate and ray relatives in which traumatic wounds heal completely and quickly. Our hypothesis is that rapid and infection-free wound healing in elasmobranch fishes is related to antimicrobial activity present in epidermal mucus and that mucus-derived compounds will be effective against a variety of wound infection pathogens. The aims of this project are to identify antimicrobial activity against wound infection pathogens by epidermal mucus secretions of stingrays and skates, to determine the contribution of mucus to the rate of healing and resistance to infection in these fish, to establish biochemical profiles for mucus protein/peptide factors and to isolate antimicrobial compounds extracted from epidermal mucus. The studies to achieve these aims are coordinated among personnel at four institutions: Mote Marine Laboratory, Daemen College Center for Wound Healing Research, University of South Florida Center for Biological Defense, and Clemson University Animal & Veterinary Sciences. The anticipated research findings will impact the CMDRP Basic Research Program **Polytrauma and Blast Injury** project tasks directed toward **Wound Infection Prevention & Management** and **Antimicrobial Countermeasures** by identifying antibiotic compounds with the potential for development into novel antimicrobial agents that will facilitate the treatment of polymicrobial infections of combat-related wounds.

BODY:

Research during Year 3 of this three-year funded project contributed information toward portions of most Tasks described in the SOW. Many of the Task objectives were planned to be continuous or on-going throughout the project. While considerable data related to each Task have been collected, objectives for some of the Tasks remain to be competed. As a result, a 1-year no cost extension was requested and granted to focus on unfinished objectives outlined in the Scope of Work.

Briefly, Project PI and Co-PI's plan to complete the originally proposed research to 1) characterize antimicrobial activity in stingray epidermal mucus, 2) isolate compounds potentially responsible for the mucus-associated antibiotic activity, 3) characterize wound healing rates, corresponding histology, and healing biomarkers, and 4) explore the role of antibiotic mucus compounds in the infection-free healing observed in these animals so that, ultimately, our wounded warriors could benefit from this promising research. During the 1-year extension, the Project PI plans to submit manuscripts for publication describing the project results.

A primary driver in the need for additional time is that more research animals (specifically Atlantic stingrays, *Dasyatis sabina*) are required to complete the wound healing histology trajectory and mucus biomarker studies. Results to date indicate exciting findings with regard to the wound healing process, which will benefit from additional time to complete a thorough investigation of these phenomena at the histological and protein biomarker levels. Greater volumes of mucus are also required in order to conduct the proposed biomarker studies and this will be accomplished through pooling of mucus samples collected from experimental wound sites of multiple animals. The ability to collect additional *D sabina* will be delayed by a few months as these animals are not readily available in the area until water temperatures warm up by late spring. Other considerations include completion of mucus compound isolation and completion of primary and secondary antimicrobial screening of mucus extracts and microbial symbionts that are still being prepared.

Research accomplishments associated with Tasks and Subtasks outlined in the proposed Statement of Work are described below.

Task 1. Collect animals and epidermal mucus

Animal Care and Use Review Office (ACURO) and Mote Marine Laboratory IACUC protocols have been reviewed, approved, and updated as necessary throughout the funded period. IACUC approvals at Mote Marine Laboratory are renewed annually, with the most recently reviewed and approved renewal occurring on 27 January, 2014.

Collection of research animals is an ongoing activity during the project, weather permitting. The original project Scope of Work included two species of stingray (*Rhinoptera bonasus* and *Dasyatis sabina*). Research results during years 1, 2, and 3 utilized these species. While the clearnose skate (*Raja eglanteria*) was planned for inclusion, they were noticeably absent until year 3 of the studies. Coincidentally, a third species of ray (*Mobula hypostoma*), whose occurrence has been historically sporadic, was regularly present during year 3 and was added to an amended Scope of Work. Also included in this report are preliminary sampling data from a freshwater population of the Atlantic stingray (*Dasyatis sabina*).

Because of their active, schooling behavior, cownose rays, *Rhinoptera bonasus*, and devil rays, *Mobula hypostoma*, are sampled passively at time of capture and released unharmed. Because of their of their sedentary and solitary behavior, clearnose skates, *Raja eglanteria*, and Atlantic stingrays, *Dasyatis sabina*, are ideal for long-term captive maintenance in smaller tanks and can be sampled multiple times. Atlantic stingrays can be easily manipulated for experimental procedures, and have been the ray of choice for experimental wounding studies conducted during Years 2 and 3.

Methods to collect epidermal mucus developed during Year 1 continue to be successful and consist of sampling individual rays by passive scraping of the pectoral fin surfaces with a sterile scoopula and transferring the mucus to sterile culture tubes. During the most recent quarter, improvements in collecting mucus from wound sites were achieved and will provide valuable data related to ongoing studies of wound healing histology trajectory and identification of wound-associated mucus biomarkers.

Task 2. Determine antimicrobial activity of epidermal mucus

Initial Characterization of Devil Ray Mucus

Historically, devil rays (*Mobula hypostoma)* inhabit local waters on an irregular basis. Since the winter of 2012, they have been regularly present in nearshore waters of the Gulf of Mexico. With the opportunity to add an additional source of epidermal mucus to the project, the Scope of Work was amended in February, 2013, to include this species. Like cownose rays, devil rays are collected passively by surrounding them in shallow water with a seine net and transferring them with dip nets to an onboard live-well, where they can be sampled at time of capture and released unharmed.

As with other rays, devil ray mucus can be separated by gentle centrifugation into an aqueous supernatant and a viscous pellet (Figure 1), although devil ray mucus is noticeably thicker than mucus from either cownose rays or Atlantic rays.

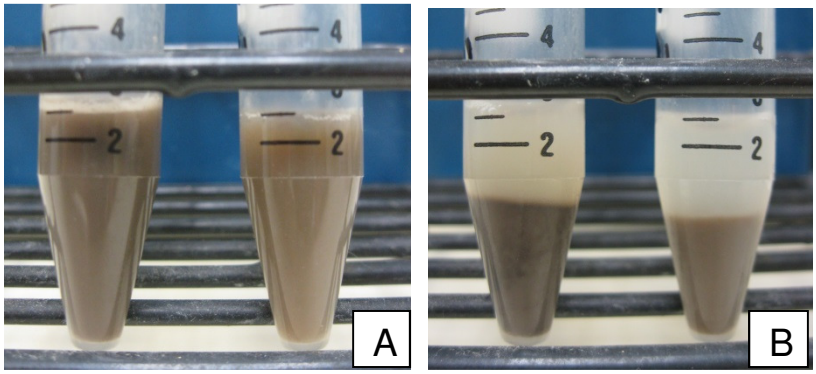


Figure 1. Fresh devil ray epidermal mucus before (A) and after (B) centrifugation, separating the sample into an aqueous supernatant and a mucus pellet.

As visualized on electrophoretic analysis using SDS-polyacrylamide gel electrophoresis (Figure 2), aqueous supernatant contains at least 20 proteins/protein subunits. As with other rays, the protein pattern for devil rays is consistent among individuals of the same species.

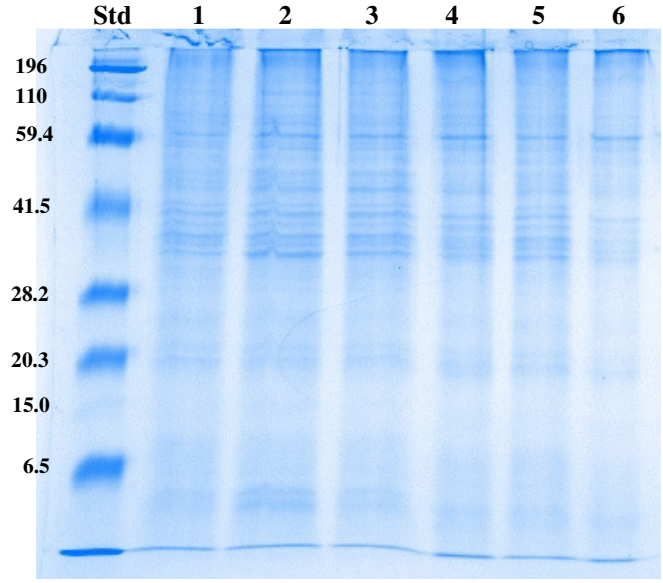


Figure 2. SDS 12% polyacrylamide gels of supernatant proteins/protein subunits from fresh mucus of six devil rays, *Mobula hypostoma*, stained with Coomassie blue. Molecular weights of standard proteins are in kilodaltons (kD).

Initial Characterization of Clearnose Skate Mucus

Fresh mucus from clearnose skates has a noticeably different consistency compared to mucus from any of the ray species. While this difference is difficult to describe, the terms “gelatinous” or “sticky” are reasonably accurate descriptors. The mucus pellet did not separate from the aqueous supernatant as readily as in ray mucus, and required additional centrifugation steps to achieve separation. Aqueous supernatant fractions of freshly obtained clearnose skate mucus also contain numerous proteins, although not in as high a concentration. For this reason, proteins/protein subunits separated using SDS-polyacrylamide electrophoresis were visualized using silver stain (Figure 3). As observed with the ray species, protein patterns for clearnose skates are consistent among individuals of the same species.

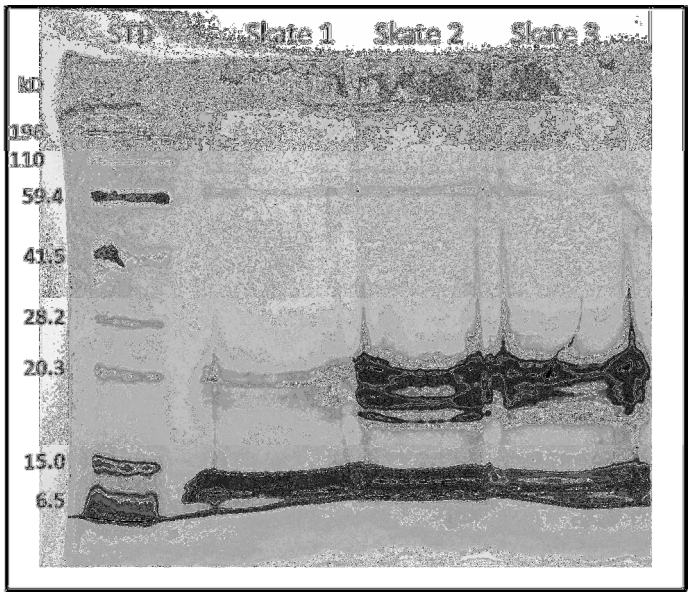


Figure 3. SDS 12% polyacrylamide gel of supernatant proteins/protein subunits from fresh mucus of three clearnose skates, *Raja eglanteria*, visualized with silver stain. Molecular weights of standard proteins are in kilodaltons (kD).

Initial Characterization of Mucus from Freshwater Atlantic Stingrays

Because of their ability to osmoregulate over a broad range of salinities, both marine and freshwater populations of the Atlantic stingray inhabit Florida waters, providing the unique opportunity to compare mucus from fresh and saltwater environments from the same species. Probably as a reflection of their tannic acid river and lake environment, mucus from the freshwater rays is noticeably darker than from marine specimens (Figure 4A and 4B). Separation of fresh mucus into aqueous supernatant and mucus pellet demonstrates that the pigment remains in the mucus pellet (Figure 4D). As visualized using electrophoresis, aqueous supernatant components are remarkably similar among mucus samples from fresh and saltwater environments (Figure 5), with a few noticeable differences in the higher molecular weight region of the gels.

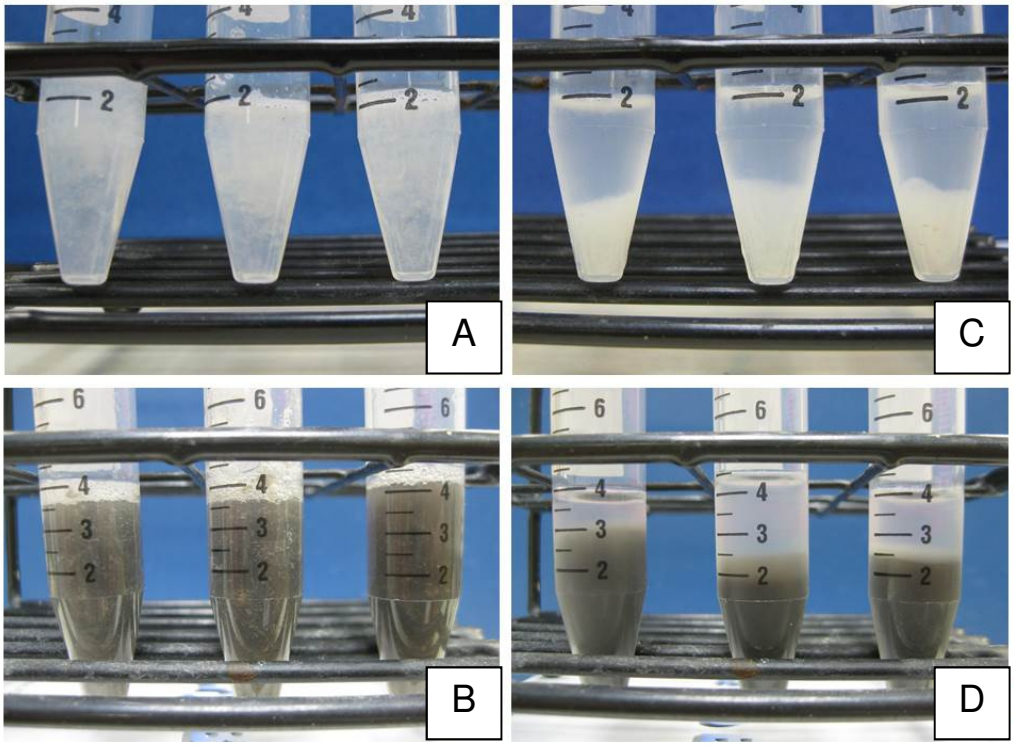


Figure 4. Comparison of fresh mucus from marine (top) and freshwater (bottom) Atlantic stingrays before (A and B) and after (C and D) centrifugation.

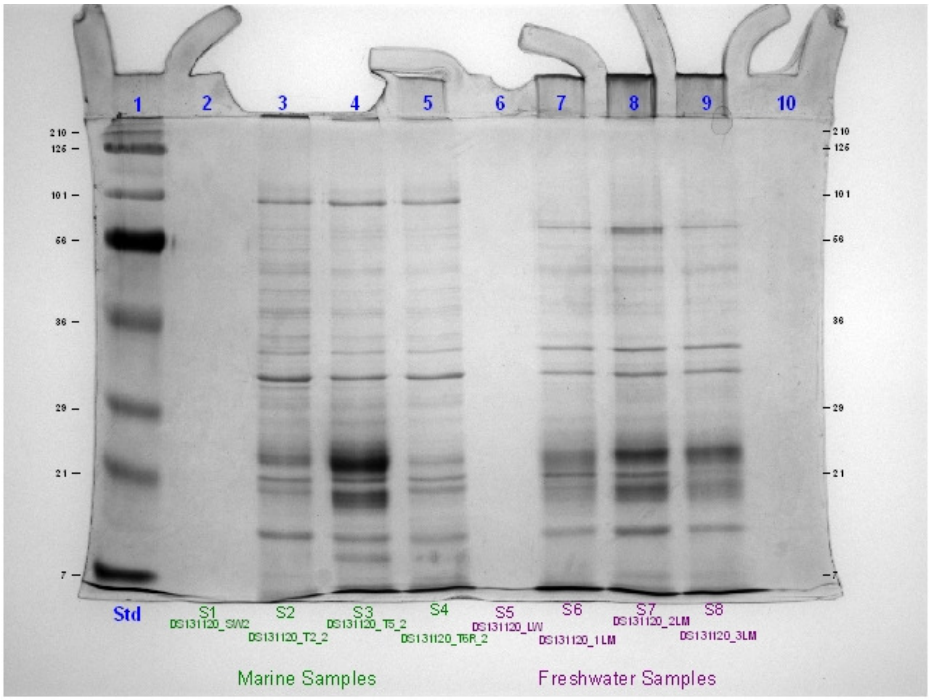


Figure 5. SDS electrophoretic gel separation comparison of aqueous supernatant proteins from mucus of marine (left) and freshwater (right) Atlantic stingrays.

Magnesium Trifluoroacetate Extraction of Cownose Ray and Devil Ray Mucus

Freshly collected mucus samples from cownose rays and devil rays were pooled, separated into aqueous supernatants and mucus pellets, combined with equal volumes of 1 M $Mg(TFA)_2$ and gently mixed on a rocking platform for a minimum of 2 hrs at 4° C. Samples were subjected to centrifugation to remove residual material and/or undissolved pellet and supernatants (extracts) were aspirated. Mucus pellets were extracted two additional times, with supernatants from the second and third pellet extractions combined and analyzed separately to compare with the initial pellet extract. Resulting supernatants were dialyzed against 0.05 M ammonium bicarbonate, using 3,500 and 1,000 MW cutoff dialysis tubing, and lyophilized. Seawater collected at the time of mucus sampling was treated in an identical fashion and used as a control for potential contribution of seawater to fresh mucus.

SDS polyacrylamide gel electrophoresis of cownose ray and devil ray mucus proteins extracted with $Mg(TFA)_2$ and dialyzed using 3,500 MW cutoff dialysis tubing are shown in Figure 6. As demonstrated previously with Atlantic stingray mucus, protein patterns reveal that not only does the extract have many of the proteins present prior to extraction, but some appear to be enriched in the mucus extracts.

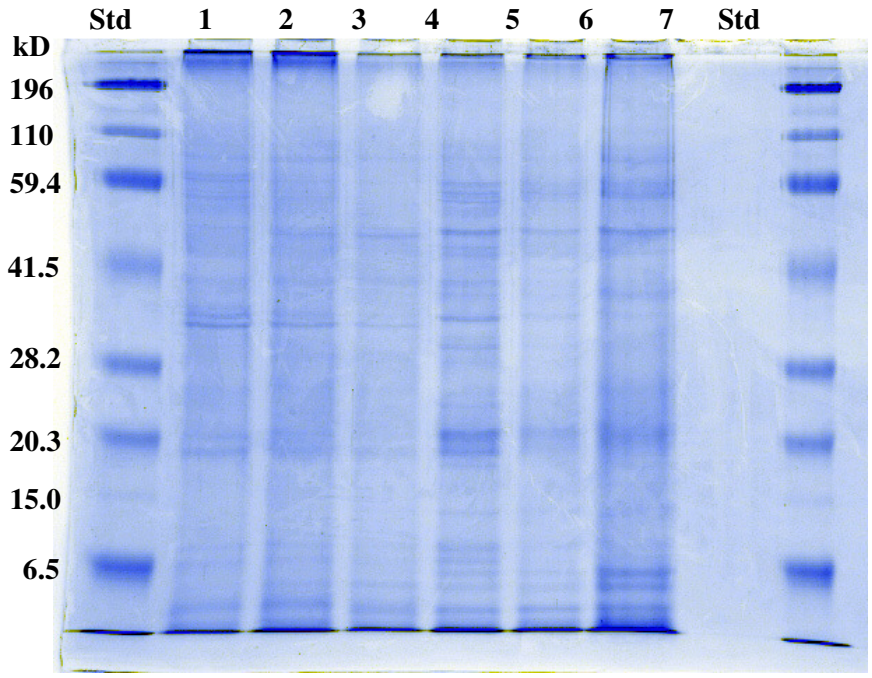


Figure 6. 12% SDS polyacrylamide gels of $Mg(TFA)_2$-extracted proteins from cownose ray, *Rhinoptera bonasus* (lanes 1-3) and devil ray, *Mobula hypostoma* (lanes 4-6) mucus. Lanes 1 and 4, 1x extracted aqueous supernatant; lanes 2 and 5, 1x extracted mucus pellet; lanes 3 and 6, 2x and 3x extracted mucus pellet; lane 7, 1x extracted seawater. Standard molecular weight proteins are in the outer lanes.

Mucus extracts were also dialyzed using 1,000 MW cutoff tubing to investigate whether small molecular weight mucus proteins/peptides are potentially lost using 3,500 MW cutoff dialysis tubing. SDS polyacrylamide gel electrophoretic profiles of $Mg(TFA)_2$-extracted proteins comparing the two molecular weight cutoffs are shown in Figure 7.

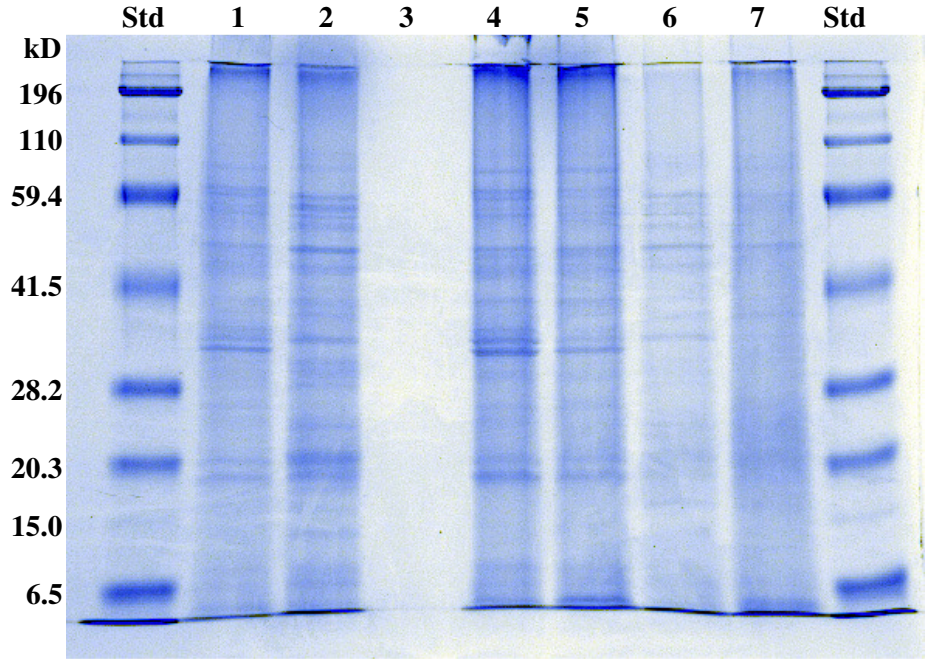


Figure 7. 12% SDS polyacrylamide gels of $Mg(TFA)_2$-extracted proteins from cownose ray, *Rhinoptera bonasus* and devil ray, *Mobula hypostoma* mucus comparing profiles following dialysis with 3,500 MW cutoff (lanes 1 and 2) and 1,000 MW cutoff (lanes 3 through 7) dialysis tubing. Lanes 1 and 2, *R. bonasus* and *M. hypostoma* aqueous supernatant; lane 3, extracted seawater; lane 4 and 5, *M. hypostoma* aqueous supernatant and mucus pellet; lanes 6 and 7, *R. bonasus* aqueous supernatant and mucus pellet. Standard molecular weight proteins are in the outer lanes.

Initial inspection of the low molecular weight region of the gels reveals no dramatic differences in banding patterns, although some of the bands near the bottom of gels appear to stain more intensely in the extracts dialyzed with the 1,000 MW cutoff tubing.

Antibiotic Activity of Magnesium Trifluoroacetate-extracted Mucus

Previous attempts to screen for antibiotic activity in fresh mucus and extracts of fresh mucus have utilized a standard turbidity assay, where the extent to which samples inhibit growth of pathogen cultures is determined spectrophotometrically as a decrease in turbidity (absorbance at 600 nm) compared with control pathogen cultures. These assays have not proven to be as useful as anticipated.

With mucus collected from cownose rays and devil rays, preliminary studies were performed to screen $Mg(TFA)_2$-extracted mucus samples for antibiotic activity using a modified Kirby-Bauer disk diffusion assay. In this assay, pathogens are grown on agar plates overlaid with filter paper disks containing mucus extracts and various activity controls. If the pathogens are susceptible to material on a particular disk, an area of clearing surrounds the disk where the pathogen is not capable of growing (called a zone of inhibition).

An example of an agar plate from preliminary disk diffusion assays against *Bacillus subtilis* is shown in Figure 8. Samples to be assayed for antibiotic activity are dissolved in a 1% DMSO PBS solution and applied to filter paper disks, which are placed on the surface of the pathogen plate. Zones of inhibition can be seen around disk number 1 and disk number 9. Disk numbers 1, 2, and 3 contain protein from $Mg(TFA)_2$-extracted devil ray mucus, disk number 7 contains $Mg(TFA)_2$-extracted seawater, disk 8 contains 1% DMSO (vehicle control), and disk number 9 contains a mixture of Penicillin and Streptomycin (positive inhibitory control).

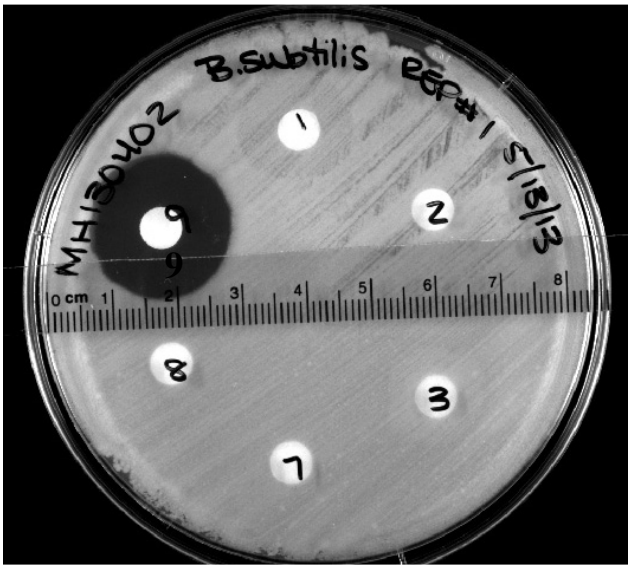


Figure 8. *Bacillus subtilis* disk diffusion assay plate. Disk 1, $Mg(TFA)_2$-extracted aqueous supernatant; Disk 2, $Mg(TFA)_2$-extracted mucus pellet; Disk 3, mucus pellet extracted with $Mg(TFA)_2$ two additional times; Disk 7, $Mg(TFA)_2$-extracted seawater; Disk 8, 1% DMSO PBS; Disk 9, Penicillin/Streptomycin mixture. Mucus assayed on this plate was collected from devil rays (*Mobula hypostoma*).

While zones of inhibition in these pilot study disk diffusion assays are faint, the application of this procedure to mucus compounds is very encouraging. Several modifications and/or refinements to improve visibility and sample application are in progress.

Task 3. Separation of bacterial symbionts from epidermal mucus

As mentioned earlier in this report, clearnose skates (*Raja eglanteria*) were absent from the area until Year 3 of the study. As a result, symbionts from freshly collected skate mucus were isolated using methods applied to cownose rays, devil rays, and Atlantic stingrays. Mucus was serially diluted in sterile seawater and plated onto marine agar (Sigma). Cultures were grown at ambient temperature for 3-5 days for development of bacterial colonies (Figure 9). Microorganisms exhibiting a unique colony or cellular morphology compared to other colonies on a single plate were subcultured to purification under the same set of growth conditions. Purified isolates were cryopreserved at -80ºC in 96-well microtiter plate "culturable" libraries and stored for antimicrobial screening.

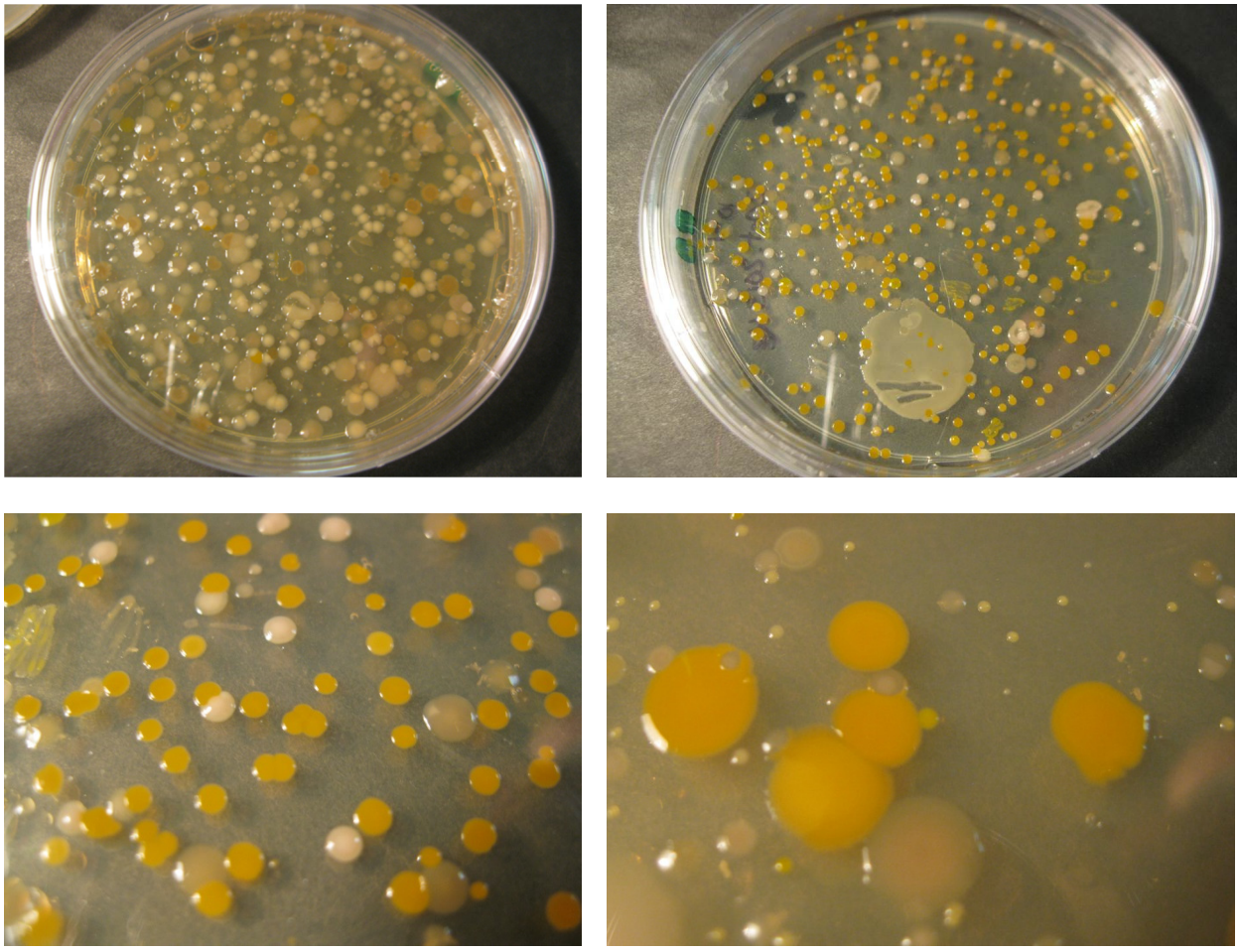


Figure 9. Representative initial cultures of bacterial symbionts isolated from freshly collected clearnose skate epidermal mucus.

Also during Year 3, mucus samples from a freshwater population of Atlantic stingrays (*Dasyatis sabina*) were obtained for initial studies. Because of their ability to osmoregulate over a broad range of salinities, both marine and freshwater populations of this species inhabit Florida waters, providing the unique opportunity to compare microbial symbionts residing in mucus from fresh and saltwater environments from the same species. Representative cultures of bacterial symbionts from freshwater Atlantic stingrays are shown in Figure 10.

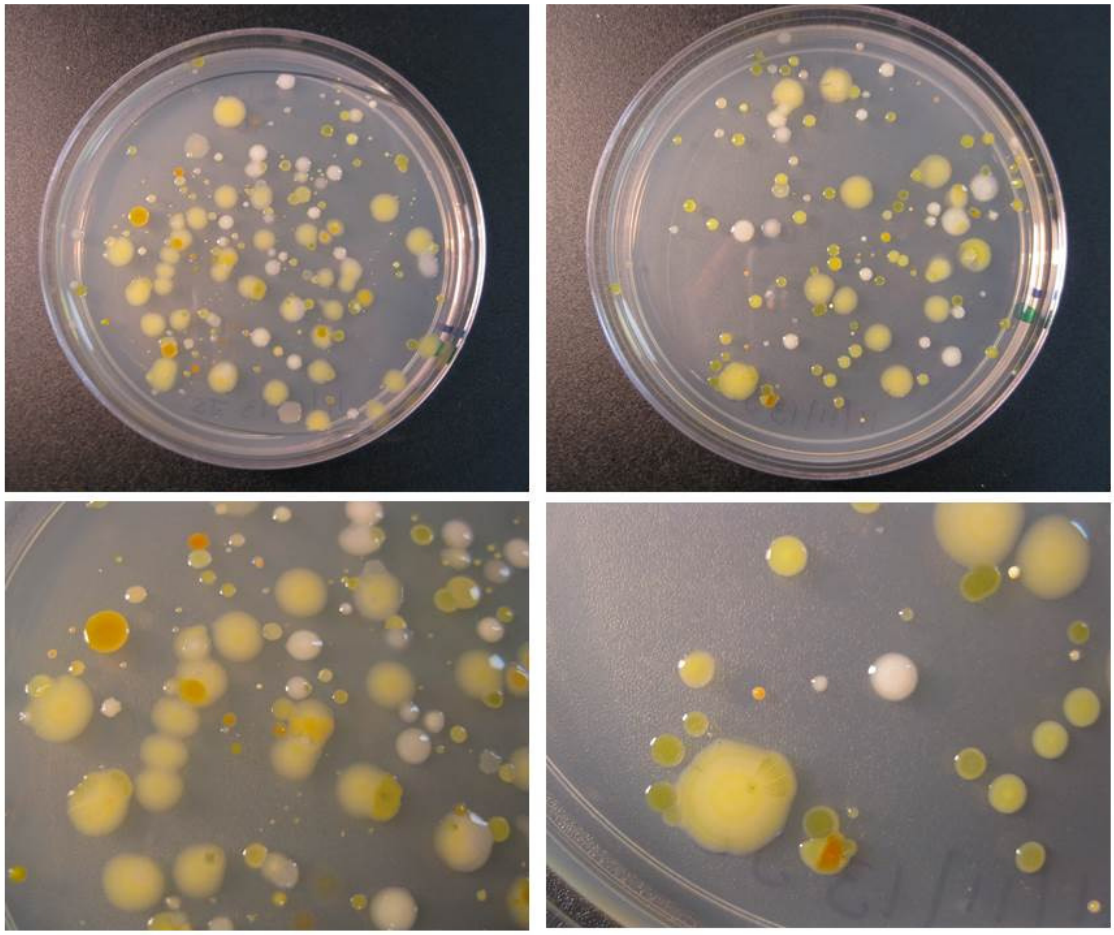


Figure 10. Representative initial cultures of bacterial symbionts isolated from freshly collected epidermal mucus from freshwater Atlantic stingrays.

Primary and Secondary Screens of Bacterial Isolates from Cownose Ray Mucus against Wound Infection Pathogens

During Year 2 Quarter 4 (Year 2 Annual Report), it was reported that 135 bacterial isolates cultured from cownose ray epidermal mucus collected in December, 2012, demonstrated antibiotic activity against at least one human pathogenic tester strain maintained at Mote Marine Laboratory (Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus*, Vancomycin resistant *Enterococcus*, *Enterococcus faecalis*, *Vibrio shiloi*, *Escherichia coli*, and *Bacillus subtilis*). Twenty-nine of these isolates were active against MRSA.

During Year 3, these 135 isolates were provided to project collaborators at the University of South Florida Center for Biological Defense (CBD) for secondary screening. During the first Quarter of Year 3, secondary screening data for 48 of the 135 strains were performed, with four strains demonstrating antibiotic activity against pathogenic bacterial strains maintained at CBD (*Bacillus cereus*, Methicillin-resistant *Staphylococcus aureus*, *Listeria monocytogenes*, *Micrococcus* sp., *Enterococcus faecalis*, *Enterococcus faecium*, Vancomycin resistant *Enterococcus*, and *Escherichia coli*). During the second Quarter of Year 3, secondary screens of the remaining 87 isolates were performed, with 10 strains demonstrating antibiotic activity against pathogenic bacterial strains maintained at CBD.

Cownose rays sampled in Year 3 Quarter 2 contributed an additional seven bacterial isolates with antibiotic activity against at least one human pathogenic tester strain maintained at MML. One of these strains showed antibiotic activity against pathogenic bacterial strains maintained at CBD. The 15 strains demonstrating antibiotic activity in BOTH primary and secondary screens are shown in Table 1.

Table 1. Bacterial isolates from cownose ray (*R. bonasus*) mucus demonstrating activity in primary AND secondary screens against pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML) and USF Center for Biological Defense (CBD).

Strain ID	Species	Primary Screen Activity (MML) plus Zone of Inhibition (mm)	Secondary Screen Activity (CBD) plus Zone of Inhibition (mm)
803 D10	*Rhinoptera bonasus*	MRSA (9.5) MSSA (4.5)	*Bacillus cereus* (6) *Listeria monocytogenes* (6) MRSA (2) *Micrococcus* sp (7) VRE (2)
803 E6	*Rhinoptera bonasus*	MRSA (7.5) MSSA (8.5) VRE (4.5) *Bacillus subtilis* (10)	MRSA (10) MRSA (10) *Micrococcus* (16) *Listeria monocytogenes* (13) VRE (4)

803 G11	*Rhinoptera bonasus*	MRSA (6) MSSA (4.5) *Bacillus subtilis* (7.5)	*Bacillus cereus* (4) MRSA (8) VRE (4) *Micrococcus* (13) *Listeria monocytogenes* (3)
804 C6	*Rhinoptera bonasus*	MRSA (1.25)	*Micrococcus* (2) *Enterococcus faecalis* (2) VRE (2) *Listeria monocytogenes* (2)
805 D2	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecalis* (2) *Listeria monocytogenes* (3)
805 D11	*Rhinoptera bonasus*	VRE (2.5) *Bacillus subtilis* (5.75)	*Listeria monocytogenes* (2)
805 D12	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecium* (10) VRE (10) *Listeria monocytogenes* (4)
805 G2	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecalis* (10) *Enterococcus faecium* (10) VRE (10) *Listeria monocytogenes* (10)
805 G4	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecalis* (10) *Enterococcus faecium* (10) VRE (10) *Listeria monocytogenes* (10)
805 H6	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecium* (5) VRE (5)
805 H7	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecium* (5) VRE (5)
806 E11	*Rhinoptera bonasus*	MSSA (1) MRSA (1) *Bacillus subtilis* (1)	*Enterococcus faecalis* (3) *Enterococcus faecium* (6) VRE (6) *Listeria monocytogenes* (6)
807 A2	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecalis* (5) *Enterococcus faecium* (3) VRE (4) *Listeria monocytogenes* (5)
807 A8	*Rhinoptera bonasus*	*Bacillus subtilis* (1)	*Enterococcus faecalis* (4) *Enterococcus faecium* (7) VRE (7) *Listeria monocytogenes* (6)
816 C8	*Rhinoptera bonasus*	MRSA (2.0) MSSA (1.0) *Bacillus subtilis* (4.0)	*Bacillus cereus* (2) *Enterococcus faecalis* (1)

Primary and Secondary Screens of Bacterial Isolates from Devil Ray Mucus

Devil rays (*Mobula hypostoma*) were added to the SOW during Year 3. Primary screening of 192 bacterial isolates from initial mucus sampling for this species resulted in 35 of the 192 isolates demonstrating antibiotic activity against at least one tester strain maintained at Mote Marine Laboratory (Table 2). Twenty-four of the purified isolates had activities against *Bacillus subtilis*, 10 were active against *Vibrio shiloi*, 2 against VRE, 4 against MRSA, and 2 against MSSA. Five isolates had activity against 2 or more tester strains. Two isolates had antibiotic activity against pathogenic bacterial strains maintained at CBD (Table 3).

Table 2. Antibiotic activity of bacterial isolates from devil ray (*Mobula hypostoma*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory

Strain ID	Antibiotic Activity plus Zone of Inhibition (mm)	Strain ID	Antibiotic Activity plus Zone of Inhibition (mm)
809 A2	*Vibrio shiloi* (0.1)	809 E6	*Bacillus subtilis* (0.1)
809 A3	*Vibrio shiloi* (0.1)	809 G5	*Bacillus subtilis* (1.0)
809 A4	*Bacillus subtilis* (0.25) *Vibrio shiloi* (4.5)	809 H6	*Bacillus subtilis* (0.1)
809 A6	*Vibrio shiloi* (0.2)	809 H9	MRSA (1.5)
809 A8	VRE (2.0)	810 D1	*Bacillus subtilis* (0.1)
809 A9	VRE (1.0) MRSA (0.75), MSSA (1.0) *Vibrio shiloi* (2.0)	810 D3	*Bacillus subtilis* (1.0)
809 B1	*Bacillus subtilis* (1.0)	810 E1	*Bacillus subtilis* (0.3)
809 B4	*Vibrio shiloi* (1.0)	810 E4	*Bacillus subtilis* (0.1)
809 B5	*Vibrio shiloi* (1.0)	810 E5	*Bacillus subtilis* (0.1)
809 B6	*Vibrio shiloi* (1.0)	810 E11	*Bacillus subtilis* (0.1)
809 B12	*Bacillus subtilis* (0.1)	810 F6	*Bacillus subtilis* (0.5)
809 C1	*Bacillus subtilis* (0.1)	810 F11	*Bacillus subtilis* (0.2)
809 C4	*Bacillus subtilis* (0.2)	810 G1	*Bacillus subtilis* (0.2) MRSA (0.1)
809 C5	*Vibrio shiloi* (1.0)	810 G5	*Bacillus subtilis* (0.1)
809 C6	*Bacillus subtilis* (0.1) *Vibrio shiloi* (0.5)	810 G11	*Bacillus subtilis* (0.2)
809 D6	*Bacillus subtilis* (0.1)	810 H1	*Bacillus subtilis* (0.1)
809 D9	MRSA (1.0), MSSA (1.0)	810 H6	*Bacillus subtilis* (0.2)
809 E5	*Bacillus subtilis* (2.0)		

Table 3. Bacterial isolates from devil ray (*Mobula hypostoma*) mucus showing activity in primary AND secondary screens against pathogenic bacterial tester strains maintained at Mote Marine Laboratory (MML) and USF Center for Biological Defense (CBD).

Strain ID	Species	Primary Screen Activity (MML) plus Zone of Inhibition (mm)	Secondary Screen Activity (CBD) plus Zone of Inhibition (mm)
809 A9	*Mobula hypostoma*	MRSA (0.75) MSSA (1.0) VRE (1.0) *Vibrio shiloi* (2.0)	*Bacillus cereus* (2) *Listeria monocytogenes* (2) *VRE (3)* *Enterococcus faecalis* (2) *Enterococcus faecium* (3)
809 D9	*Mobula hypostoma*	MRSA (1.0) MSSA (1.0)	*Enterococcus* sp (1) *VRE (4)* *Enterococcus faecalis* (2) *Enterococcus faecium* (4)

Primary Screens of Bacterial Isolates from Clearnose Skate Mucus

Mucus from clearnose skates (*Raja eglanteria*) was sampled for the first time during Year 3. A total of 288 isolates was generated from clearnose skate mucus, with seven isolates demonstrating antibiotic activities (Table 4). All seven were active against *E. coli*; one isolate was also active against MRSA.

Table 4. Antibiotic activity of bacterial isolates from clearnose skate (*Raja eglanteria*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory

Strain ID	Primary Screen Activity + Zone of Inhibition (mm)
842 C11	*Escherichia coli* (4.0)
842 D5	*Escherichia coli* (3.0)
842 E4	*Escherichia coli* (2.0)
842 E5	*Escherichia coli* (3.0)
842 E8	*Escherichia coli* (4.0)
842 E9	*Escherichia coli* (4.0), MRSA (4.0)
842 E11	*Escherichia coli* (3.0)

Primary Screens of Bacterial Isolates from Freshwater Atlantic Stingray Mucus

As mentioned earlier, mucus samples from a freshwater population of Atlantic stingrays (*Dasyatis sabina*) were obtained for initial studies. A total of 96 isolates was generated from the freshwater ray mucus, with nine isolates demonstrating antibiotic activities (Table 5). All nine were active against *E. coli*; one isolate was active against MSSA.

Table 5. Antibiotic activity of bacterial isolates from freshwater Atlantic stingray (*Dasyatis sabina*) mucus against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory

Strain ID	Primary Screen Activity + Zone of Inhibition (mm)
845 FW1	*Escherichia coli* (3.0)
845 FW2	*Escherichia coli* (3.0)
845 FW3	*Escherichia coli* (2.0)
845 FW4	*Escherichia coli* (2.0)
845 FW5	*Escherichia coli* (2.0)
845 FW6	*Escherichia coli* (3.0), MSSA (5.0)
845 FW7A	*Escherichia coli* (2.0)
845 FW7B	*Escherichia coli* (2.0)
845 FW8	*Escherichia coli* (2.5)

Initial Characterization of Hemolytic Activity and Susceptibility to Proteinase K by Selected Active Isolates

Preliminary characterization of ten mucus-derived bacterial isolates demonstrating antibiotic activity against pathogens in either or both primary and secondary screens was performed using blood agar assays for presence of hemolytic activity and Proteinase K assays for peptide derived antibiotic activity (see Appendix 4 for methods). Presence of hemolytic activity could imply potentially harmful effects toward mammalian blood cells, while Proteinase K inhibition of antibiotic activity could imply that the active compound(s) are proteins. Either property would limit the viability as a systemic antibiotic compound, although they would still have potential as topical agents. Results are summarized in Table 6.

Six of the isolates displayed hemolytic activity, while four appeared to be inhibited by Proteinase K. One isolate (803 G11) was neither hemolytic activity nor inhibited by Proteinase K, suggesting that compounds responsible for antibiotic activity of this isolate would be worth further investigation.

Table 6. Hemolytic activity and susceptibility to Proteinase K for selected mucus-derived bacterial isolates

Strain ID	Antibiotic Activity plus Zone of Inhibition (mm)	Blood Agar Result plus Zone of Inhibition (mm)	Susceptibility to Proteinase K
803 A6	MRSA (2.5) *Bacillus subtilis* (3.0)	Hemolytic (2)	Not Inhibited
803 D10	MRSA (9.5) MSSA (4.5) VRE (2.0)	Hemolytic (<1)	Not Inhibited
803 E6	MRSA (7.5) MSSA (8.5) VRE (4.5) *Bacillus subtilis* (10.0)	Hemolytic (3)	Not Inhibited
803 G11	MRSA (6.0) MSSA (4.5) VRE (4.0) *Bacillus subtilis* (7.5)	Not Hemolytic	Not Inhibited
804 D4	MRSA (3.0) MSSA (1.5) *Bacillus subtilis* (3.0)	Hemolytic (1)	Inhibited
805 D11	VRE (2.5) *Bacillus subtilis* (5.75) *Listeria monocytogenes* (2.0)	Hemolytic (1)	Not Inhibited
806 E8	MRSA (1.0) MSSA (1.5) *Bacillus subtilis* (6.5)	Not Hemolytic	Inhibited
809 A9	VRE (1.0) MRSA (0.75), MSSA (1.0) *Vibrio shiloi* (2.0) *Listeria monocytogenes* (2)	Not Hemolytic	Inhibited
809 D9	MRSA (1.0) MSSA (1.0) VRE (4.0)	Not Hemolytic	Inhibited
816 C6	MRSA (2.0) MSSA (1.0) *Bacillus subtilis* (5.0)	Hemolytic (<1)	Not Inhibited

Preliminary identification of the selected bacterial isolates assayed for hemolytic activity and Proteinase K susceptibility was performed based on 16S rDNA sequence analysis. DNA was isolated from purified isolates and sequences were prepared as described in Appendix 5. GenBank BLAST searches on the generated sequences were performed in order to demonstrate % identity to known bacteria available in the worldwide database. These identities are summarized in Table 7. GenBank BLAST searches identified seven different genera among the 10 bacterial isolates.

Table 7. Bacterial identities of mucus-derived bacterial isolates based on 16S rDNA sequence analysis.

Strain ID	16S Identification (no. base pairs queried)
803 A6	*Bacillus anthracis* 99% (772)
803 D10	*Bacillus thuringiensis* 100% (812)
803 E6	*Bacillus anthracis* 99% (732)
803 G11	*Bacillus marisflavi* 99% (836)
804 D4	*Bacillus thuringiensis* 99% (865)
805 D11	*Bacillus thuringiensis* 99% (796)
806 E8	*Pseudoalteromonas issachenkonii 99% (891)*
809 A9	*Pseudoalteromonas marina* 99% (778)
809 D9	*Bacillus gibsoni* 100% (763)
816 C6	*Bacillus cereus* 99% (514)

Task 4. Determine contribution of epidermal mucus to wound healing in elasmobranchs

As demonstrated during Year 2, the Atlantic stingray, *Dasyatis sabina*, is the ray of choice for experimental wounding studies related to Aim 2 (Task 4) and Aim 3 (Task 5). Because of their size (smaller than cownose rays and devil rays) and their sedentary and solitary behavior (not found in large schools like cownose rays and devil rays), Atlantic stingrays are ideal for long-term captive maintenance in smaller tanks compared to those required for cownose rays and devil rays, and can be easily manipulated for experimental procedures.

Based on pilot studies and input from collaborators at Daemen College, it was determined that circular wounds would be the best to analyze. Using autoclaved circular brass coring tools, circular wounds penetrating the epidermal and dermal layers of the skin can be inflicted, exposing the underlying epaxial musculature (Figure 11).

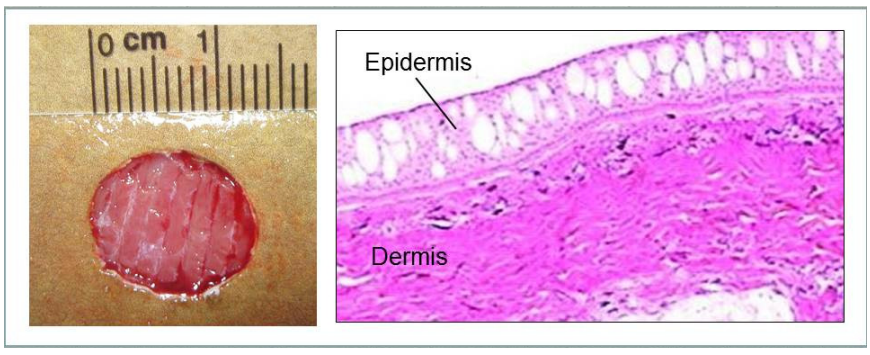


Figure 11. Experimental wounds are circular excisions of the epidermal and dermal layers of skin, exposing the dorsal epaxial musculature on the pectoral fins of stingrays.

Studies correlating gross physical changes with tissue histology

As described in Quarterly Reports, experimental wounding studies have documented the physical appearance of wounds during the progression of wound healing with digital images. Using circular wounds as described above, a reproducible timetable for the occurrence of gross physical changes was established. Following the initial formation and breakdown of superficial clotted blood, the progression of events is the formation of what appears to be a thin layer of connective tissue during the first 10 to 14 days, followed by the emergence after approximately 3 weeks of a slightly raised area in the center of wounds that appears to be fibrous in nature. This central raised area gradually flattens and spreads to the wound margins, giving the appearance of uniform healing by 8 weeks, except for lack of pigmentation.

To correlate the histological changes associated with the gross physical events, an experimental wounding study performed during Year 3 Quarter 2 was designed to biopsy wounds at intervals to bracket the appearance and spreading of the central tissue mass. Four 1.0 cm circular wounds were inflicted on an Atlantic stingray (Figure 12) with wounds biopsied at 17, 21, 24, and 28 days into the healing process. Biopsied tissues were fixed in 10% formalin and sent to Daemen College for histological processing.

Figure 12. Atlantic stingray showing the placement of four experimental circular wounds, each measuring 1 cm in diameter.

Figure 13 shows the four wounds on the day of their biopsies. As expected from the timeline previously established from physical appearance of wounds, the epaxial musculature of the day 17 wound appears to be covered by a thin layer of connective tissue. By day 21, the anticipated central tissue mass is clearly present, with gradual spreading underway in the day 24 wound. By day 28, most of the tissue mass has spread to the margins of the wound.

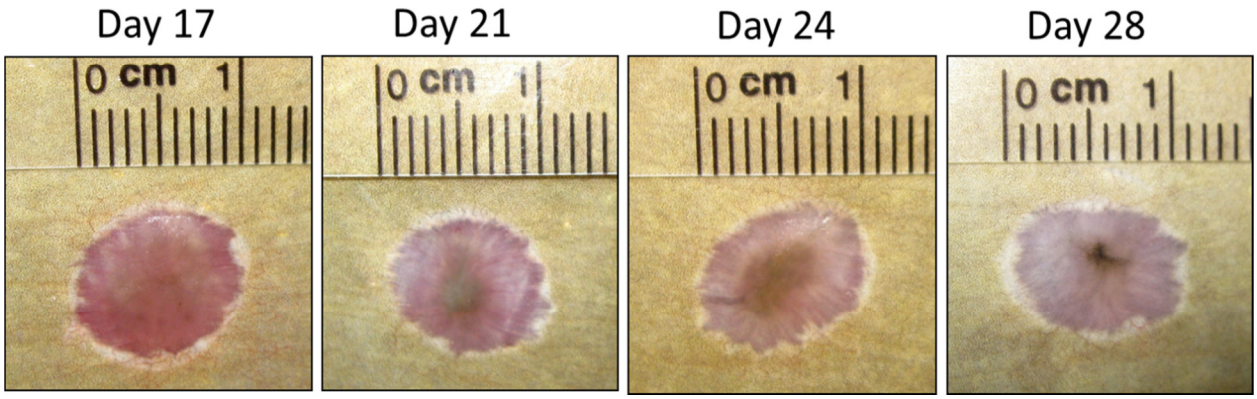


Figure 13. Experimental wounds in Figure 9, showing their physical appearance on the day of their biopsies.

The resulting histology provided unexpected information. The layer of tissue covering the epaxial musculature on day 17 was not connective tissue, but developing epidermal and dermal layers (Figure 14a). Although noticeably thick, the epidermal layer already has a spinous layer with keratinocytes and contains irregularly spaced mucus cells, mostly at or near the surface. The dermal region is in the process of restructuring and contains cells at lower than normal density. At this early phase of histological analysis, it is difficult to determine whether the appearance of raised tissue is a manifestation of the thickened epidermal and dermal layers at this stage of reformation, or whether interstitial swelling in the restructuring layers is causing the developing layers to be pushed toward the surface.

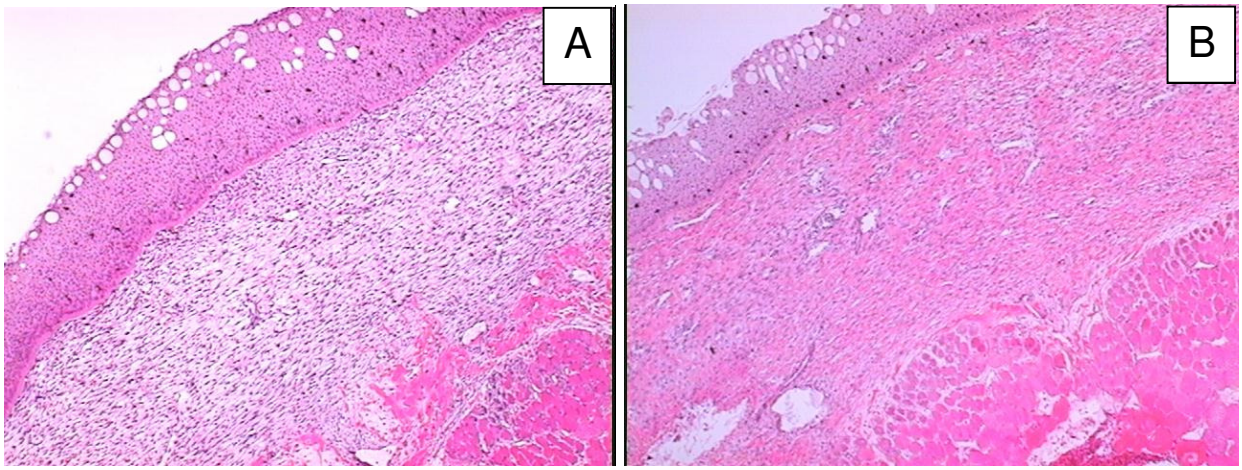


Figure 14. Gross physical appearance and corresponding tissue histology of experimental wounds biopsied on day 17 (**A**) and day 28 (**B**).

By 28 days, the thickness of the epidermis is reduced and the density of cells in the dermal region has increased with more evidence of restructuring (Figure 14B). While the physical appearance of the wounds suggested healing was in early phases by day 17, the histology showed that considerable reforming of skin layers, especially the epidermis, was well underway by this time. Subsequent experimental wounding studies examined histological changes in wounds biopsied at earlier time points in the healing process.

During Year 3 Quarter 3, an experimental wounding study in which wounds were biopsied at intervals of 2, 4, 6, 8, 10, and 12 days was performed. Physical appearance of wounds on the days they were biopsied are shown in Figure 15. As before, biopsied wounds were fixed in 10% formalin and sent to Daemen College for histological processing.

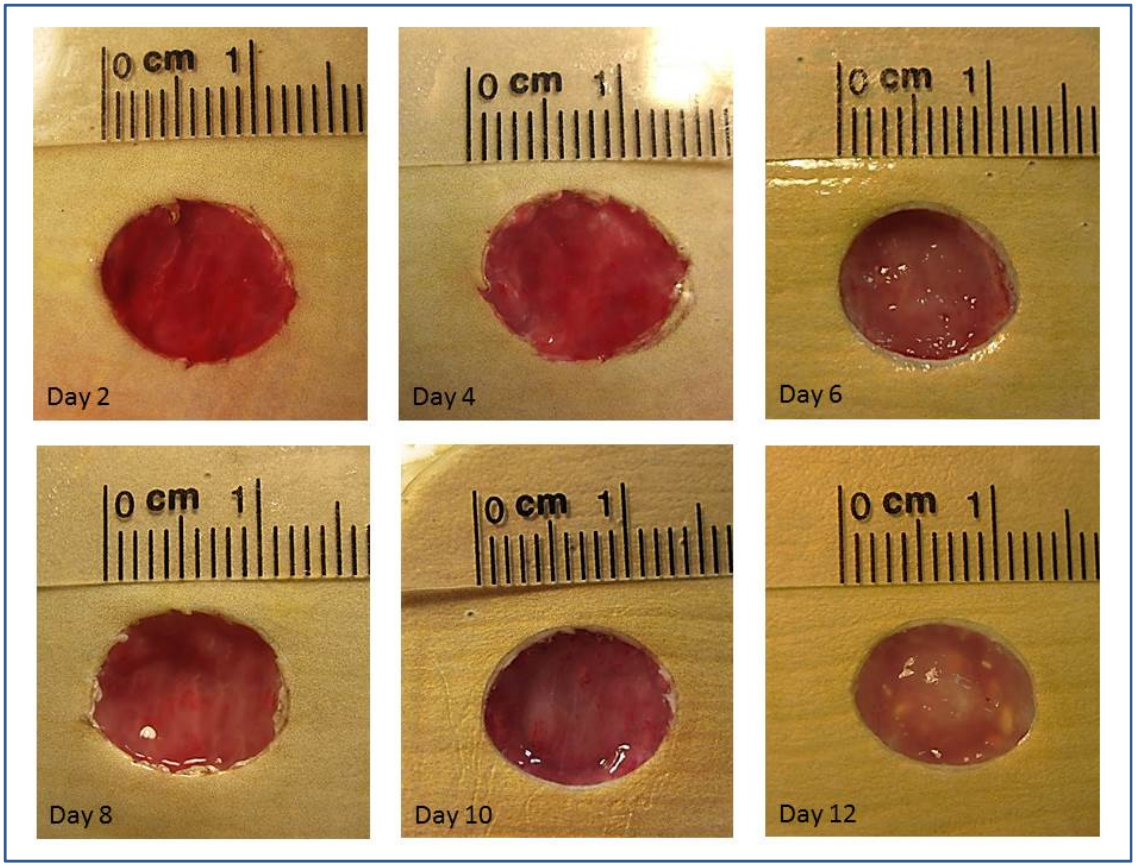


Figure 15. Physical appearance of wounds at time of biopsy showing the progression of healing from 2 days to 12 days after infliction of wounds.

Once again, histology of the wounds revealed that the restructuring of epidermal and dermal layers was occurring much earlier than expected. The Day 2 wound bed was already covered with a thin epithelium containing mucus cells and a clearly identifiable basal layer (stratum basale) (Figure 16). The developing dermis is unorganized.

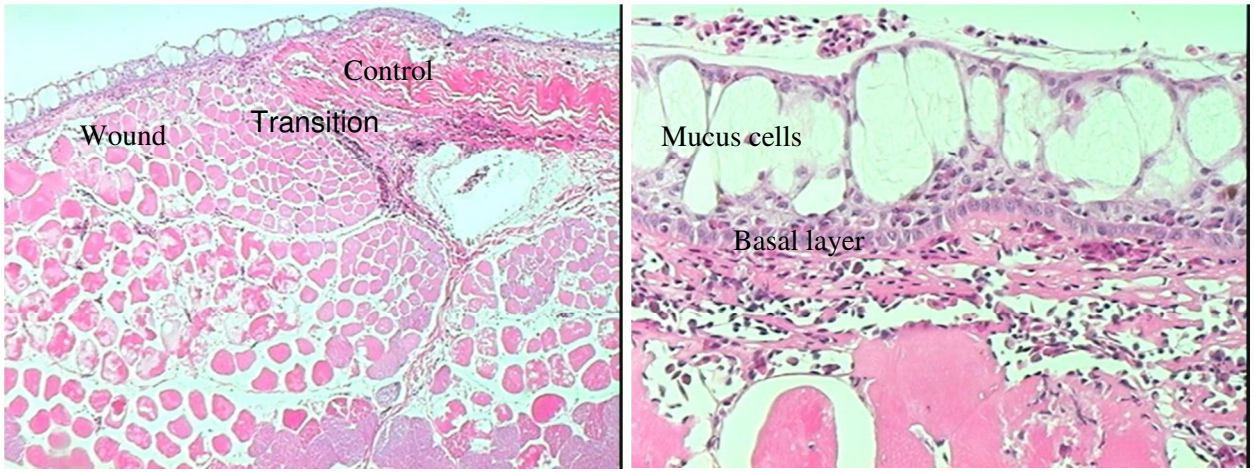


Figure 16. Tissue histology of experimental wound biopsied on Day 2, showing the presence of mucus cells in a thin epithelial layer. Left) Low power (4x) view showing the Day 2 wound, control region adjacent to the wound and the transition region. Right) Higher power (20x) view of mucus cells and basal layer in the newly formed epithelium.

By Day 6, the epidermis is noticeably thicker with an increase in mucus cells and keratinocytes. The dermis is thicker than at Day 2, but is very vascular and remains without normal architecture (Figure 17).

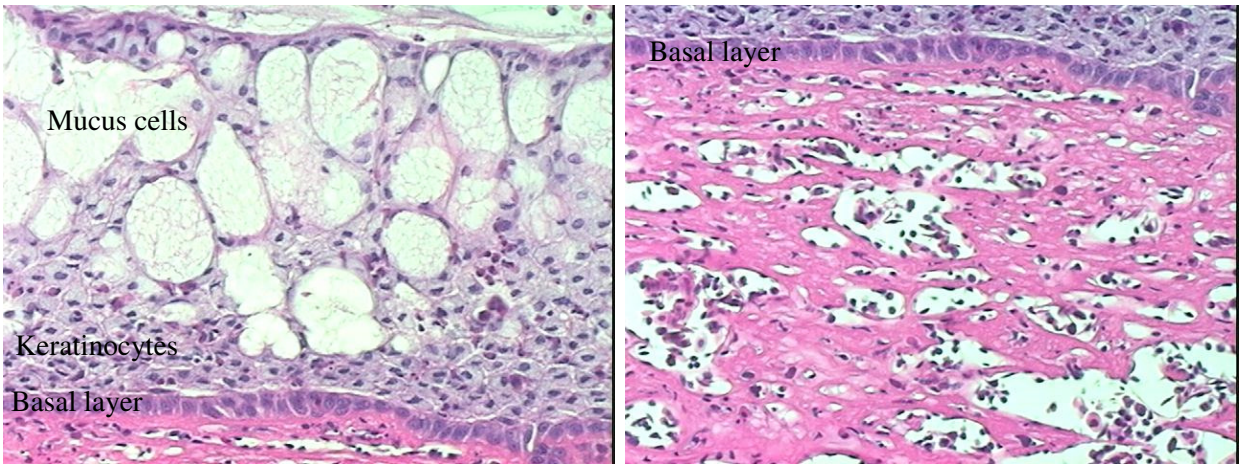


Figure 17. Tissue histology of experimental wound biopsied on Day 6 showing the increase in mucus cells and keratinocytes in the epidermal layer (left). The restructuring dermis is noticeably thicker and very vascular (right). Magnification: 20x.

Because of the appearance of an epidermal layer at Day 2 (48 h), experimental wounding studies to examine histological changes in wounds biopsied at even earlier time points in the healing process were performed during Year 3 Quarter 4. Experimental wounds were inflicted and biopsied at 8 h, 16 h, and 24 h, fixed in 10% formalin, and sent to Daemen College for histological processing.

These recent studies helped to demonstrate the importance of the first two days in the early phases of the healing process. At 8 hours (Figure 18), the wound bed is overlaid

with what appears to be a protective blood clot. There is a distinct transition where the wound margin meets the surrounding undisturbed epidermal and dermal layers.

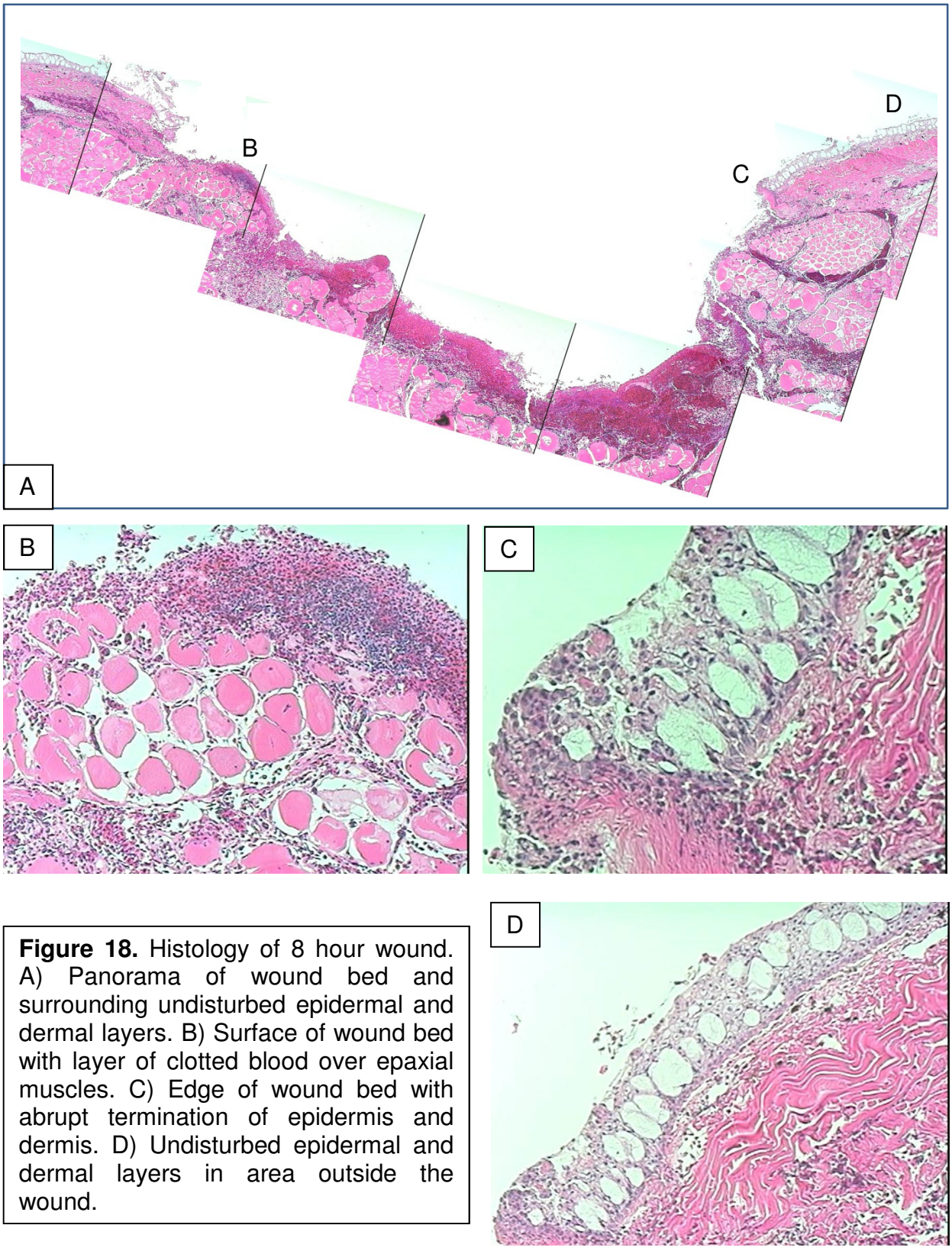
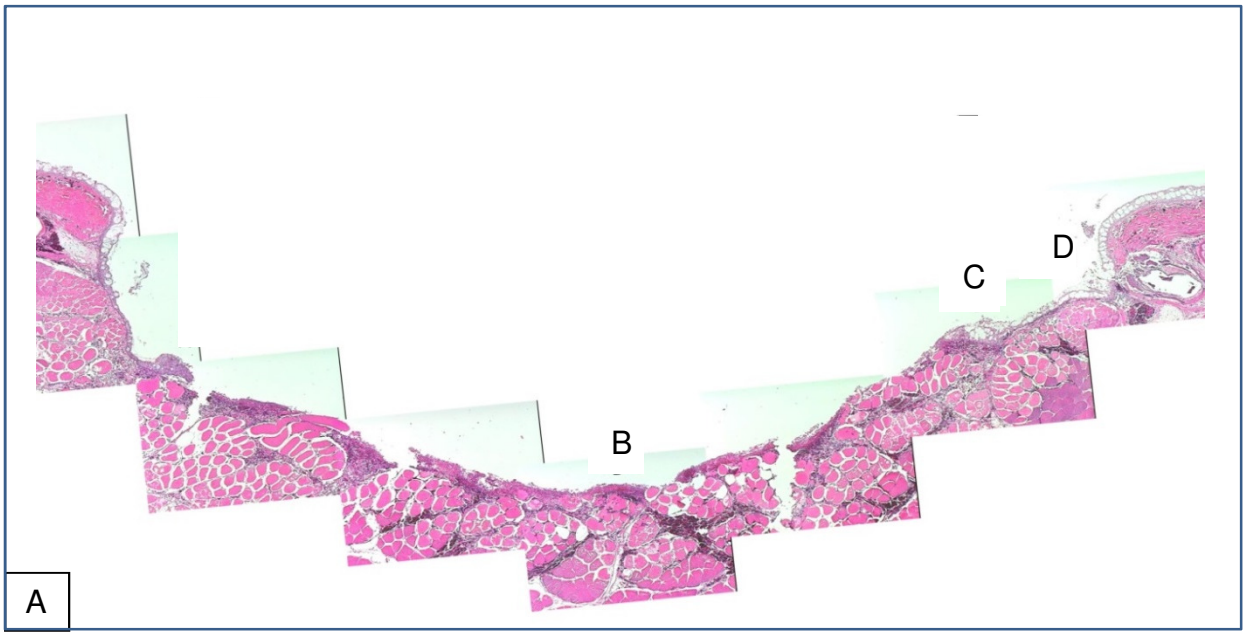
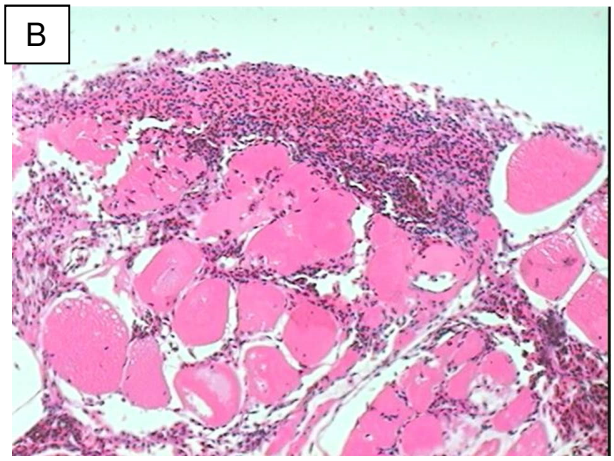
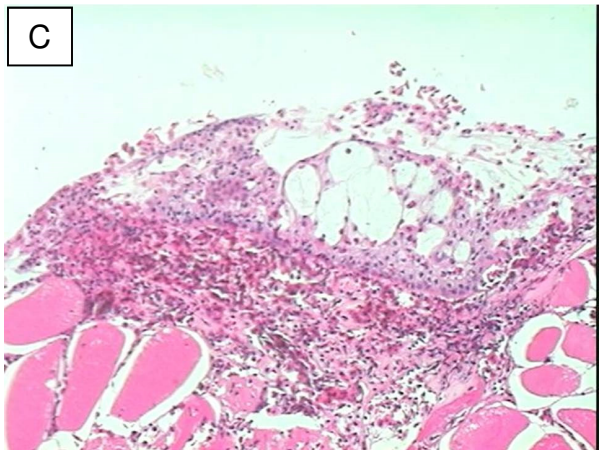


Figure 18. Histology of 8 hour wound. A) Panorama of wound bed and surrounding undisturbed epidermal and dermal layers. B) Surface of wound bed with layer of clotted blood over epaxial muscles. C) Edge of wound bed with abrupt termination of epidermis and dermis. D) Undisturbed epidermal and dermal layers in area outside the wound.

By 24 hours (Figure 19), the blood clot is reduced and the epidermal layer is beginning to infiltrate the surface of the wound bed from the periphery of the wound, with mucus cells migrating onto the wound from the surrounding undisturbed area.







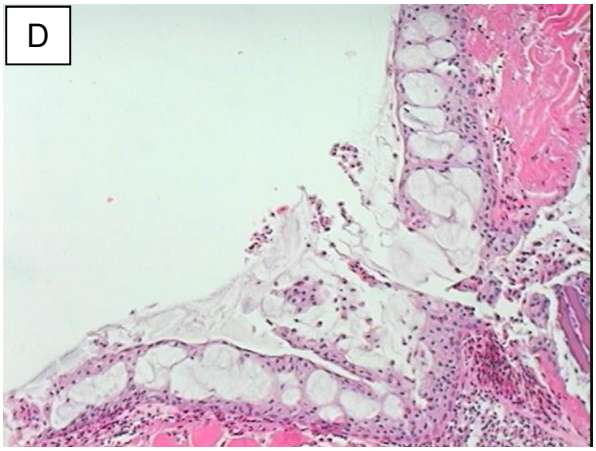


Figure 19. Histology of 24 hour wound. A) Panorama of wound bed and surrounding undisturbed epidermal and dermal layers. B) Middle of wound bed surface with patch of residual blood clot. C) Infiltration of epidermal layer across the wound bed surface. D) Migration of epidermal mucus cells from areas outside the wound margin onto the surface of the wound bed.

Task 5. Determine changes in biochemical profiles associated with wound healing

With the histology providing evidence that an initial migration of epidermal cells across the surface of the wound could be seen by 24 hours and that by 48 hours the wound bed was already covered with a thin epithelium containing mucus cells and a clearly identifiable basal layer (stratum basale), it was obvious that wound-associated mucus samples during this key time period would be necessary for the evaluation of biochemical profiles associated with wound healing.

In ongoing studies initiated during Year 3 Quarter 4, mucus samples from wound bed surfaces and from non-wounded control areas were collected at 24 and 48 hours after wounding (Figure 20).

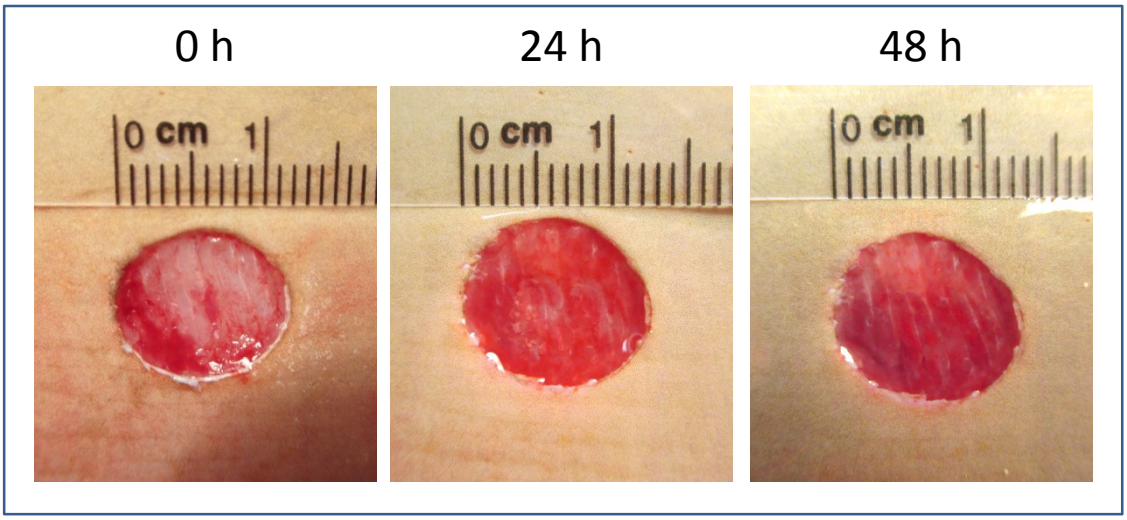


Figure 20. Representative physical appearance of wounds at 0, 24, and 48 hours showing the clot-covered wound bed from which mucus associated with early-phase healing was collected.

To obtain adequate volumes of material, multiple animals were wounded. Approximately 50 to 100 µL samples of mucus were collected from the surface of wound beds. Because of the viscous nature of the mucus, samples were minimally diluted with sterile elasmobranch-modified PBS (phosphate buffered saline) and homogenized. Samples were centrifuged at 12,000 x *g* for 10 minutes, supernatants were aspirated, and protein determinations were performed. Total protein ranged from about 400 to 1200 µg in 24 hour samples and from 500 to 1,000 µg in 48 hour samples. Due to the variable presence of blood clots at these early time periods, the inclusion of blood clot-associated proteins in the mucus samples, as inferred from the slight pinkish color, was unavoidable. Following the addition of protease inhibitors, samples were separated into 3 to 5 aliquots of 50 to 100 µL and frozen immediately at -80° C in preparation for shipment to Daemen College, where biomarker protein arrays and iTRAQ analysis of proteins/peptides will be conducted.

Task 6. Isolate bioactive compounds in epidermal mucus.

Separation of Compounds from Magnesium Trifluoroacetate Extracted Devil Ray Mucus

Freshly collected mucus samples from devil rays were pooled, separated into aqueous supernatants and mucus pellets, combined with equal volumes of 1 M $Mg(TFA)_2$ and gently mixed on a rocking platform for a minimum of 2 hours at 4° C. Samples were subjected to centrifugation to remove residual material and/or undissolved pellet and supernatants (extracts) were aspirated. Mucus pellets were extracted two additional times, with supernatants from the second and third pellet extractions combined and analyzed separately to compare with the initial pellet extract. Resulting supernatants were dialyzed against 0.05 M ammonium bicarbonate using 1,000 MW cutoff dialysis tubing and lyophilized. Seawater collected at the time of mucus sampling was treated in an identical fashion and used as a control for potential contribution of seawater to fresh mucus. Lyophilized extracts were sent to collaborators at Clemson University chromatographic separation of component proteins.

At Clemson, lyophilized mucus extracts were brought up in 1 mL of 0.025 M Histidine (start buffer for Chromatofocusing), centrifuged, then assayed for protein at 280 nm. Protein was 6.75 mg/mL. The diluted (1/20) aliquot for protein analysis was run using gel filtration chromatography (Figure 21), while the rest of the sample was applied to a chromatofocusing column using 0.025 M Histidine pH 6.4 as start buffer and PB 74/HCl 1:8 pH 4.2 as running buffer (Figure 22). Fractions collected were assayed for protein at 280 nm.

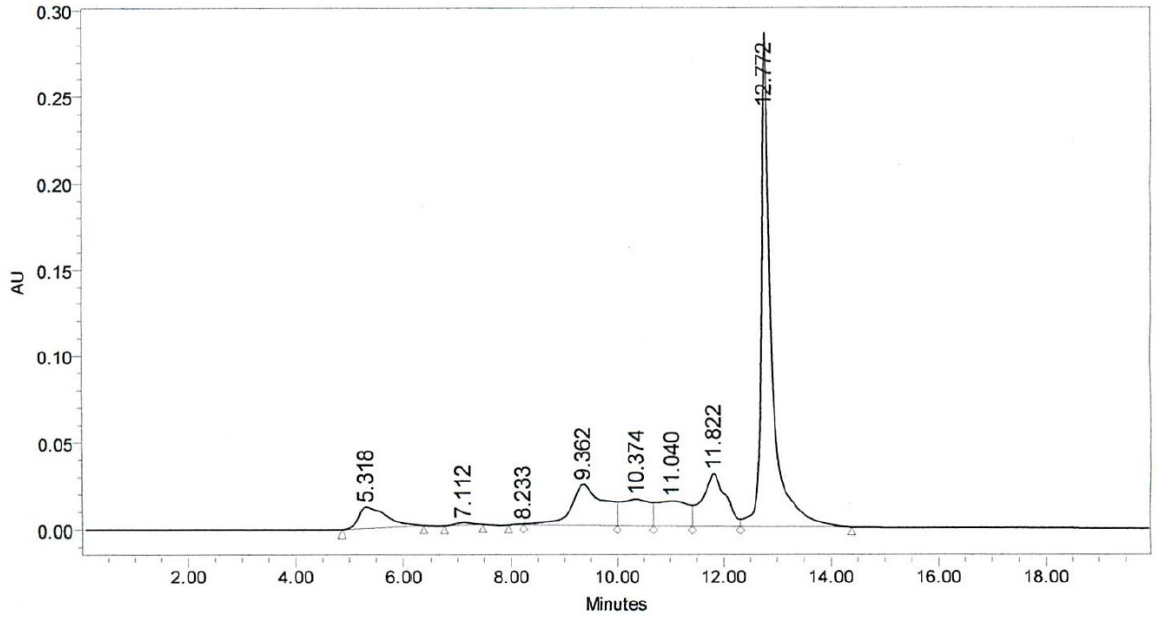


Figure 21. Separation of mucus extract on a Waters 600 HPLC system using a Biosep SEC-2000 300x7.8 (Phenomenex) gel filtration column. Injection volume, 100 µL; Run time, 20 min; Mobile phase, 0.05M Phosphate, 0.15M NaCl, pH 7.0; Flow rate, 1.0 mL/min; Detection, 280 nm.

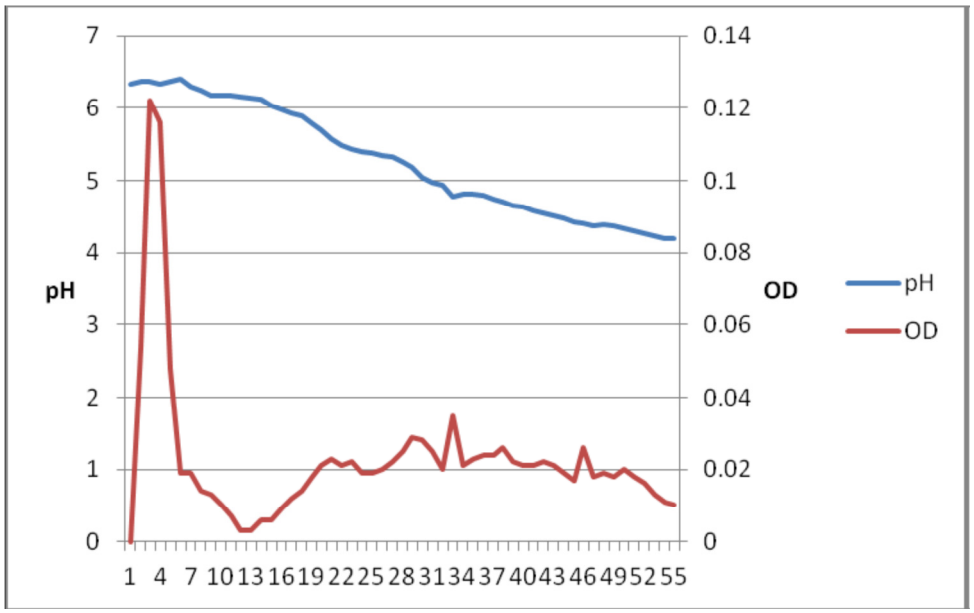


Figure 22. Separation of mucus extract on a Pharmacia K9/15 chromatofucusing column using PBE 94:PB 74-HCl Polybuffer exchanger (GE Healthcare) diluted 1:8 with dH20 and adjusted to pH 6.4 with HCl. Running buffer, 0.025 Histidine – HCl, pH 4.2; Bed volume, 5.03 cm^3.

Fractions 2-4 and 18-52 from the chromatofocusing column were pooled and separated using gel filtration (Figure 23) and SDS polyacrylamide gel electrophoresis (Figure 24).

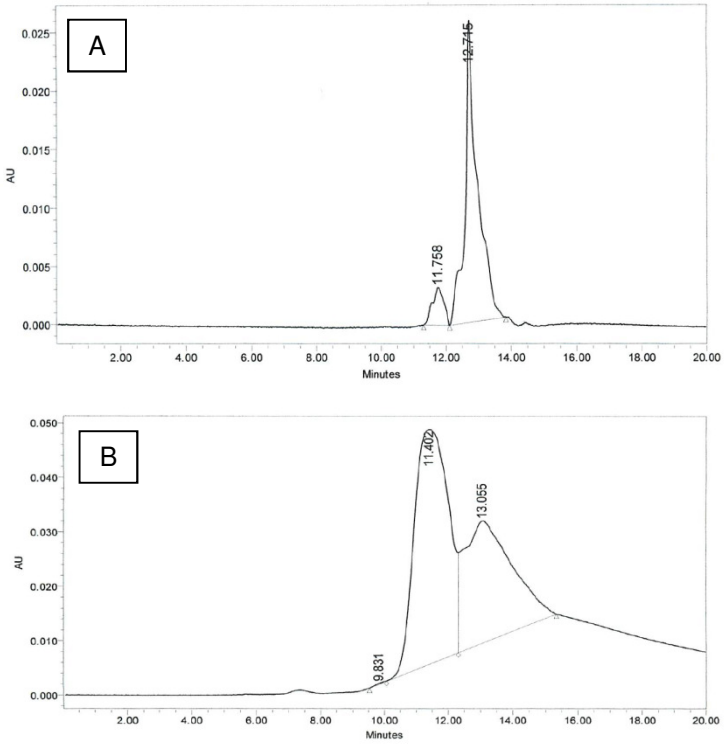


Figure 23. Gel filtration separation of pooled fractions 2-4 (A) and pooled fractions 18-52 (B) eluted from the chromatofocusing separation shown in Figure 19. Chromatographic column and separation conditions are as described in the legend to Figure

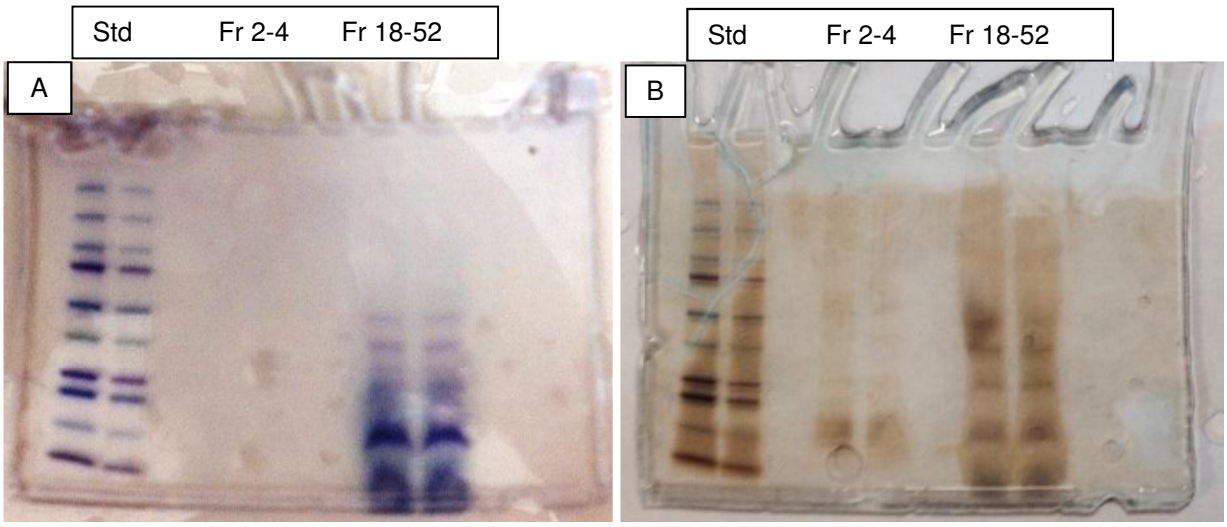


Figure 24. SDS polyacrylamide gel electrophoretic separation of pooled fractions 2-4 and pooled fractions 18-52 eluted from the chromatofocusing separation shown in Figure 7. **A)** Stained with Coomassie blue. **B)** Stained with silver stain. Gels were 4-20% gradient gels using a Biorad Mini-PROTEAN TGX electrophoresis system.

KEY RESEARCH ACCOMPLISHMENTS:

- Magnesium trifluoroacetate extracts of mucus pellets enriched in low molecular weight compounds result in low but measurable antibiotic activity using a modified Kirby-Bauer disk diffusion assay. Extracted compounds are being separated using HPLC and chromatofocusing.

- 192 bacterial isolates were purified from devil ray (*Mobula hypostoma*) epidermal mucus and culturable libraries have been cryopreserved. In primary screens performed at Mote Marine Laboratory, 35 of the 192 strains showed antibiotic activity against at least one human pathogenic tester strain. Twenty-four of the purified isolates had activities against *Bacillus subtilis*, 10 were active against *Vibrio shiloi*, 2 against VRE, 4 against MRSA, and 2 against MSSA. Five isolates had activity against 2 or more tester strains. Two of the 35 isolates demonstrated antibiotic activity in secondary screens against pathogenic bacterial strains maintained at University of South Florida Center for Biological Defense.

- 15 bacterial isolates purified from cownose ray (*Rhinoptera bonasus*) epidermal mucus demonstrated antibiotic activity in primary screens performed at Mote Marine Laboratory AND in secondary screens against pathogenic bacterial strains maintained at University of South Florida Center for Biological Defense. 14 of these strains were active against either MRSA or VRE.

- GenBank BLAST searches identified seven different genera among 10 bacterial isolates that produced antibacterial compounds against various tester strains and that were partially characterized by their hemolytic activity and susceptibility to Proteinase K.

- Histology performed on biopsied experimental wounds revealed that the “healing” of wounds with regard to restructuring of epidermal and dermal layers is much more rapid than suggested by the physical appearance. During the first 24 hours, an epidermal layer is infiltrating the surface of the wound bed from the periphery of the wound, and by 48 hours, wound beds are already covered with a thin epithelium containing mucus cells .

REPORTABLE OUTCOMES:

Presentations

- Poster presentation; Daniel Merselis (student presenter), Valerie Lapacek, Carl Luer, Kimberly Ritchie (co-presenter). "Antibiotic-Producing Bacteria Associated with the Epidermal Mucus of the Cownose Ray (*Rhinoptera bonasus*)." Annual Meeting of the American Society for Microbiology Florida Branch, 13-14 April, 2013, Islamorada, Florida.

- Oral presentation; Carl Luer (presenter), Cathy Walsh, Courtney Bennett, Jennifer Wyffels, Laura Edsberg. "Experimental Wounding and Preliminary Characterization of the Healing Response in the Atlantic Stingray, *Dasyatis sabina*." Annual Meeting of the American Elasmobranch Society (with 2013 Joint Meeting of Ichthyologists and Herpetologists). Albuquerque, NM, 10-15 July 2013.

- Poster presentation; Kimberly Ritchie (co-presenter), Carl Luer (co-presenter), Cathy Walsh, Jennifer Yordy, Courtney Bennett, Vicki Luna. "Antibiotic Properties Associated with Epidermal Mucus of Two Ray Species, Atlantic Stingray (*Dasyatis sabina*) and Cownose Ray (*Rhinoptera bonasus*)." Military Health System Research Symposium (MHSRS). Ft Lauderdale, FL. 12-15 August 2013.

Databases

- The worldwide database GenBank is used for BLAST searches performed on 16S rDNA sequence data generated from stingray epidermal mucus.

Conclusions

Epidermal mucus collected passively from the surface of stingrays contains proteins in both an aqueous supernatant portion and in a viscous pellet that separates from the aqueous portion upon sitting or via centrifugation. A magnesium salt of trifluoroacetic acid was successful in obtaining mucus extracts enriched in low molecular weight compounds with low but measurable antibiotic activity. High performance liquid chromatography (HPLC) and chromatofocusing columns have separated the extracted compounds based on size and isoelectric point.

While fresh and salt extracted mucus possess low but measurable antimicrobial activity, many of the symbiotic bacteria cultured from the mucus continue to demonstrate antibiotic activity in primary screens against a panel of pathogenic bacterial tester strains maintained at Mote Marine Laboratory and secondary screens against a different panel of human pathogenic tester strains maintained at University of South Florida Center for Biological Defense.

During Year 3, devil rays (*Mobula hypostoma*) were added to the SOW. 192 bacterial isolates were purified from freshly collected devil ray epidermal mucus. In primary screens, 35 of the 192 strains showed antibiotic activity against at least one human pathogenic tester strain. Twenty-four of the purified isolates had activities against *Bacillus subtilis*, 10 were active against *Vibrio shiloi*, 2 against Vancomycin resistant *Enterococcus* (VRE), 4 against Methicillin-resistant *Staphylococcus aureus* (MRSA), and 2 against Methicillin-sensitive *Staphylococcus aureus* (MSSA). Five isolates had activity against 2 or more tester strains. Two of the 35 isolates demonstrated antibiotic activity in secondary screens against pathogenic bacterial strains maintained at University of South Florida Center for Biological Defense.

Cownose ray (*Rhinoptera bonasus*) epidermal mucus continues to be the source of promising antibiotic compounds. During Year 3, bacteria isolated from cownose ray mucus generated 15 bacterial strains with antibiotic activity in primary AND secondary screens against pathogenic bacterial strains. 14 of these strains were active against either MRSA or VRE.

Experimental wounding studies during Year 3 began to correlate tissue histology of wounds with their physical appearance (i.e., the reproducible timetable for the occurrence of gross physical changes established during Year 2), with the unexpected realization that the “healing” of wounds with regard to restructuring of epidermal and dermal layers is much more rapid than suggested by the physical appearance. Several wounding studies with progressively shorter durations between infliction and biopsy of wounds revealed that during the initial 24 hours, an epidermal layer is beginning to infiltrate the surface of the wound bed from the periphery of the wound, with mucus cells migrating onto the wound from the surrounding undisturbed area. By 48 hours, wound beds are already covered with a thin epithelium containing mucus cells and a clearly identifiable basal layer (stratum basale).

Evaluation of the knowledge:

Since this funded program focuses on basic research, development of a medical "product" is not within the scope of this project. However, the antimicrobial activity demonstrated by numerous mucus-associated bacterial isolates holds great promise for the identification of antibiotic compounds and future development of therapeutics to treat wounds sustained on the battlefield. Further characterization of the remarkably rapid epidermal covering of wound beds observed during experimental wounding studies will provide valuable insight for improved treatment of wounds and wound healing.

APPENDICES:

Appendix 1. Preparation of 1 M magnesium trifluoroacetate

Appendix 2. Procedure for antibiotic screening of cultured libraries (Mote Marine Laboratory)

Appendix 3. Procedure for antibiotic screening of cultured libraries (University of South Florida Center for Biological Defense)

Appendix 4. Procedures for blood agar and Proteinase K Assays

Appendix 5. DNA extraction, amplification and sequencing of 16S subunits

Appendix 6. Meeting abstracts

1) Annual Meeting of the American Society for Microbiology Florida Branch, Islamorada, Florida, 13-14 April, 2013.

2) Annual Meeting of the American Elasmobranch Society (with 2013 Joint Meeting of Ichthyologists and Herpetologists). Albuquerque, NM, 10-15 July 2013.

3) Military Health System Research Symposium (MHSRS). Ft Lauderdale, FL. 12-15 August 2013.

Appendix 1. Preparation of 1 M magnesium trifluoroacetate

In a fume hood, place 50 mL of water in a 250mL beaker and support beaker in ice. After about 10 minutes, slowly and carefully add 15.2 mL (22.8 grams) of trifluoroacetic acid. The solution will fume vigorously. After 5 minutes, add a total of 4 grams of magnesium oxide in 4 or 5 equal portions with swirling or magnetic stirring. The reaction is mild if the temperature remains below 5° C. After the addition of the MgO, allow the reaction mixture to warm to room temperature with stirring. When the temperature is approximately room temperature, dilute to near 100 mL, check the pH, and adjust with small portions of MgO or trifluoroacetic acid until the pH is between pH 7 and pH 8. (Note: it usually takes ~0.1 gram of MgO to bring the pH to slightly less than 8.) Filter the solution. This will produce 0.1 mole of $Mg(TFA)_2$ in 100 mL of water = 1 M.

Appendix 2. Procedure for antibiotic screening of cultured libraries (Mote Marine Laboratory):

Bacteria are plated from libraries onto rectangular single well plates containing GASWA, Marine Agar, or other appropriate media. At least 2-3 days growth is required at room temperature to grow organisms to sufficient sized "colonies" for assays. The night before antibiotic assays are to be performed, cultures of tester strains are started. Overnight growth is done at 37ºC with gentle agitation.

On the day of (or the day before) the assays, rectangular plates with cultured libraries are UV-irradiated to kill the colonies being tested. This eliminates cross-contamination when overlaying with the tester strains.

0.8% agar overlays (LB, TSB, GASW and marine broth overlays) for tester strains are prepared, autoclaved, and placed in an incubator at 42ºC with gentle agitation.

Place plates (without lid) in the hood and turn on the UV lamp. Irradiate on high for 15-30 minutes (UV resistance may vary depending on the source of bacteria used in library generation). Mark the plates that are irradiated in some way with a marker to indicate they have been "killed."

1. Assay Set Up:
    a) Remove appropriate agar from incubator
    b) Inoculate with appropriate amount of batch culture (amounts may vary depending on growth stage).

2. Using glass pipette transfer 10ml of 0.8% agar from container to one well plate, move back and forth along center or empty edge of plate dispensing agar, then tilt the plate to distribute, ensure complete covering. Have appropriate tubes, UV irradiated plates, and pipettes warm in the 42ºC incubator.

3. Incubate library plates overnight at 30º C.

4. Identify zones of inhibition and note width from edge of colony using calipers. Record data for each strain in Excel file using frozen storage library grid. Data entered include tester strain active against and diameter in mm of zone of inhibition for each tester strain noted.

Appendix 3. Procedure for antibiotic screening of cultured libraries (University of South Florida Center for Biological Defense)

All isolates were subbed onto marine agar (MA), tryptic soy agar supplemented with 5% sheep red blood cells (BA), thiosulfate citrate bile salts sucrose agar (TCBS), and plain tryptic soy agar (TSA) and incubated for 2 days or longer (up to 5 days) until growth was evident. Growth was quantified and described. Gram stains were made using a P-swab to transfer bacteria to glass slides. Each specimen was methanol-fixed and left to dry. Slides were stained with crystal violet for 1 minute, rinsed with water, saturated with iodine for 1 minute, rinsed with water, de-stained with ethanol for 3 seconds, rinsed with water, and counterstained with safranin for 1 minute. In addition, KOH testing was also performed by placing a loopful of bacterial growth into a drop of 3% potassium hydroxide on a glass slide and mixed thoroughly for 60 seconds. A positive KOH reaction (signifying a Gram-negative bacterium) was demonstrated by the mixture becoming viscous and when the loop was lifted, the mixture formed a string extending from the slide to the loop.

Depending upon the Gram-stain/ KOH reaction, either MEP plates or API biochemical kits were used to further characterize the isolates. API panels were set up per manufacturer's instructions and incubated at 30º C for 24-48 hours (until 3 positive tests occurred). We did not expect to identify the gram negative isolates but wanted more information for each of them. We also wanted to rule out any *Vibrio* isolates since these came from a marine environment. MEP plates were used to help classify the Gram positive bacteria as *Bacillus* spp or not, or members of the *Bacillus cereus* group. Optimal temperatures (25, 30 or 35ºC) were determined for all isolates. Isolates were streaked onto marine agar slants for immediate use and for later use, suspended in marine broth supplemented with 25% glycerol and frozen at -80℃.

For antimicrobial screening, the marine isolates were grown on marine agar for two days at room temperature. Suspensions of these were made in 1mL of marine broth and adjusted to equal a 2 McFarland standard. Then 100 µL of each suspension was placed into a well of a microtiter plate. Using a 48 prong frogger, the bacterial suspensions were inoculated onto marine agar media plates. Orientation of each plate was noted by use of a crystal violet spot in one or two of the wells. Plates were left upright (media side down) for one hour at room temperature, then turned over and left to incubate for 48 hours. In the meantime, testing strains were grown on BA media and checked for purity and subbed so testing would use 24 hour growth.

On testing day, the marine agar plates with the marine isolate spots were placed into the biosafety cabinet media side down and the lids taken off. The growth was exposed to UV light for 45 minutes and the plates were then closed and ready for use. One to five colonies of the testing strains were used to inoculate demineralized water and adjusted to match a 1 McFarland standard. Then 100 µL of that suspension was placed into 20 mL of melted (and cooled to 55-60℃) marine agar and slowly mixed by inversion two times. The agar/bacteria mixture was slowly poured over the marine agar plates containing the dead marine isolate spots and allowed to cover evenly and cooled.

When the plates were cooled and the agar solidified, another layer of uninoculated tryptic soy agar was poured over the agar/bacteria layer and again, the plates were allowed to cool. After 45 minutes, the plates were inverted and incubated at 30℃ overnight.

The next day, the plates were examined for zones of inhibition of growth of the testing strains. The zone diameters were measured. No activity of the marine isolate products against the testing strain was. Activity was described as any zone of no growth above and around the marine isolate colony spot.

Appendix 4. Procedures for blood agar and Proteinase K Assays

Zobell marine agar plates are made with 5% sheep's blood by volume. Individual colonies of each isolate are inoculated onto a blood agar plate. After overnight growth, radii of hemolytic zones are measured in millimeters and recorded. Areas in which the red coloration of the blood agar turn transparent signify hemolytic colonies, while areas retaining red coloration signify non-hemolytic colonies.

For Proteinase K analysis, colonies are inoculated individually onto marine agar. After two days of growth, colonies are overlaid with either MRSA or *B. subtilis* using the protocol described in Appendix 2, with the addition of 100 µg of Proteinase K per milliliter of .8% agar. If zones of inhibition were present after a day of growth, Proteinase K did not inhibit the antimicrobial action of the isolate.

Appendix 5. DNA extraction, amplification and sequencing of 16S subunits

DNA was extracted from pure cultures of each colony using a Power Soil DNA Extraction Kit (Mo Bio), as per Mo Bio extraction protocol. Extracted DNA was run on a low annealing temperature polymerase chain reaction (PCR) cycle in a 25 µL reaction mixture: 12.5 µL Taq MM (Qiagen), 1.0 µL U9F universal primers, 1.0 µL U1509R universal primers, 1.0 µL bovine serum albumin, 8.5 µL molecular grade water, and 1.0 µL template DNA. These specific primers were used to isolate the 16S subunit sequence. PCR products were identified using 1% agarose gel electrophoresis and purified using a Qiagen PCR purification kit. The purified products were subsequently sequenced by UIUC Core Sequencing Facility at the University of Illinois, Urbana-Champaign.

Appendix 6. Meeting abstracts

1) Annual Meeting of the American Society for Microbiology Florida Branch, 13-14 April, 2013, Islamorada, Florida.

Antibiotic-Producing Bacteria Associated with the Epidermal Mucus of the Cownose Ray (*Rhinoptera bonasus*). Daniel Merselis (student presenter), Valerie Lapacek, Carl Luer, Kimberly Ritchie (co-presenter).

The evolution of antibiotic resistance challenges our ability to control pathogenic bacteria and increases demand for novel antibiotic discovery. This demand is compounded by decreased rates of discovery from traditional terrestrial sources. Significant marine biodiversity and corresponding antibacterial activity remain poorly described, however, and may provide a source of antibiotics in the future. In this study, we examined a novel target for bio prospecting: the symbiotic bacterial community residing in the epidermal mucus of the cownose ray (*Rhinoptera bonasus*). Five hundred and seventy-six bacterial isolates purified from freshly collected *R. bonasus* mucus were tested against a range of human and marine pathogens. One hundred and thirty-five isolates were shown to produce antibacterial properties. Of these 135, 29 exhibited activity against Methicillin-resistant *Staphylococcus aureus* (MRSA), 3 exhibited activities against Vancomycin-resistant *Enterococcus* (VRE), and 13 exhibited activities against Methicillin-sensitive *S. aureus* (MSSA). In 25 isolates, activity was demonstrated against multiple tester strains, indicating the potential production of a broad-spectrum antibiotic.

2) Annual Meeting of the American Elasmobranch Society (with 2013 Joint Meeting of Ichthyologists and Herpetologists). Albuquerque, NM, 10-15 July 2013.

Experimental Wounding and Preliminary Characterization of the Healing Response in the Atlantic Stingray, *Dasyatis sabina*. Carl Luer (presenter), Cathy Walsh, Courtney Bennett, Jennifer Wyffels, Laura Edsberg. Annual Meeting of the American Elasmobranch Society (with 2013 Joint Meeting of Ichthyologists and Herpetologists). Albuquerque, NM, 10-15 July 2013.

Wounds are commonly observed in elasmobranch fishes, yet evidence of infection, necrotic tissue and keloid scarring are notably absent. The protective secretion produced by epidermal mucus cells in Atlantic stingrays (*Dasyatis sabina*) is being investigated to identify mucus-associated antimicrobial compounds and their potential role in the infection-free healing of wounds. Circular wounds 1.0 cm in diameter were inflicted, penetrating the epidermal and dermal layers of the skin, and exposing the underlying epaxial musculature. At regular intervals, digital photographs were taken to document the gross physical changes associated with the progression of wound healing. In all experimental wounding studies to date, infection-free healing in the form of scar tissue across the wound is effectively complete in approximately six weeks. With visual inspection and digital photography, a remarkably consistent observation is the

appearance of slightly raised fibrous tissue in the center of wounds after approximately three weeks of healing which gradually flattens and spreads to the wound margins, forming uniform scar tissue across the wound. Preliminary histological examination of biopsied wounds and surrounding tissue after eight weeks of healing indicates that the wound epidermis remains distinct from the unwounded skin with respect to its pigmentation and allows the wound margin to be identified in the fixed tissue and prepared histological sections. While the wound is not fully restructured with pigmented cells, mucus cells are abundant in the hypertrophied epidermis. Wound studies designed to characterize histological changes and healing biomarkers associated with the formation and dissipation of the centralized fibrous tissue are underway.

3) Military Health System Research Symposium (MHSRS). Ft Lauderdale, FL. 12-15 August 2013.

Antibiotic Properties Associated with Epidermal Mucus of Two Ray Species, Atlantic Stingray (*Dasyatis sabina*) and Cownose Ray (*Rhinoptera bonasus*). Kimberly Ritchie (co-presenter), Carl Luer (co-presenter), Cathy Walsh, Jennifer Yordy, Courtney Bennett, Vicki Luna.

Sharks and rays recover from wounds penetrating the epidermis and dermis, with anecdotal reports of inflammation-free and infection-free healing. Secretions produced by epidermal mucus cells of two ray species (Atlantic stingray, *Dasyatis sabina* and cownose ray, *Rhinoptera bonasus*) are being investigated for antimicrobial properties with potential to identify novel antibiotic compounds to combat wound infection pathogens. Mucus consists of a protein-rich aqueous fraction and a viscous pellet with symbiotic bacteria. Chemical extraction of mucus results in partial purification of compounds with low but measurable antimicrobial activity. Antibiotic activity can be visualized as zones of growth inhibition surrounding cultured mucus-associated bacterial isolates overlaid with pathogenic bacterial tester strains. Strains at Mote Marine Laboratory (MML) include *B. subtilis*, *E. coli, E. faecalis*, VRE, MSSA and MRSA. Besides MRSA, *E. faecalis* and VRE, additional strains at USF Center for Biological Defense (CBD) include *B. anthracis*, *B. cereus, Micrococcus* sp, *S. epidermidis, E. faecium, L. monocytogenes, Shigella* sp, *P. aeruginosa, S. enterica, and Acinetobacter* sp. In primary screens at MML, 181 bacterial isolates cultured from mucus of cownose rays and 60 from Atlantic stingrays demonstrated antibiotic activity against at least one human pathogenic tester strain, while 17 cownose ray isolates and 6 Atlantic stingray isolates were actively inhibitory in secondary screens at CBD.